Institute for Reference Materials and Measurements

ERM European Reference Materials

CERTIFICATION REPORT

Certification of the Equivalent Spherical Diameters of Silica Nanoparticles in Aqueous Solution

Certified Reference Material ERM®-FD304

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The mission of the JRC-IRMM is to promote a common and reliable European measurement system in support of EU policies.

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CERTIFICATION REPORT

Certification of the Equivalent Spherical Diameters of Silica Nanoparticles in Aqueous Solution

Certified Reference Material ERM®-FD304

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Disclaimer

Certain commercial equipment, instruments, and materials are identified in this report to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the European Commission, nor does it imply that the material or equipment is necessarily the best available for the purpose.

Summary

This report describes the certification of several equivalent spherical diameters of silica nanoparticles in aqueous solution, Certified Reference Material (CRM) ERM-FD304®. The CRM has been certified by the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE.

ERM®-FD304 consists of silica nanoparticles suspended in an aqueous solution. The intended use of this CRM is to check the performance of instruments and methods that determine the diameter of nanoparticles suspended in a liquid medium. The CRM is available in 10 mL pre-scored amber glass ampoules containing approximately 9 mL of suspension.

The CRM was prepared from a commercially available colloidal silica, Ludox®, grade TM-50 (Grace Davison GmbH, Worms, Germany).

Production of the CRM included testing of the homogeneity and stability of the ampouled diluted raw material, as well as the characterisation using an interlaboratory comparison approach.

The CRM has been certified for the scattering intensity based harmonic mean diameter using Dynamic Light Scattering (DLS) and for the extinction intensity based modal Stokes diameter of the silica nanoparticles in aqueous solution using Centrifugal Liquid Sedimentation (CLS) – line start method. An indicative value for size measurements using electron microscopy and information values for pH and ζ-potential were assigned as well. Expanded uncertainties are estimated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) with a coverage factor of $k = 2$, corresponding to a level of confidence of about 95 %.

Certified values

	Equivalent spherical diameter	
	Certified value [3] [nm]	Uncertainty [4] [nm]
Scattering intensity-weighted harmonic mean diameter [1]	42.1	0.6
Extinction intensity-based modal Stokes diameter [2]	33.0	3.0

1) As obtained by dynamic light scattering according to ISO 22412:2008, Particle size analysis -- Dynamic light scattering (DLS) applying the method of cumulants as described in ISO 13321:1996, Particle size analysis - Photon correlation spectroscopy

2) As obtained by centrifugal liquid sedimentation according to ISO 13318-1:2001, Determination of particle size distribution by centrifugal liquid sedimentation methods (line-start method) and using an effective particle density of 2.305 g/cm^3.

3) Unweighted mean value of the means of accepted sets of data each set being obtained in a different laboratory and with the method of determination indicated in the respective line of the table. The certified value and its uncertainty are traceable to the International System of Units (SI).

4) The certified uncertainty is the expanded uncertainty with a coverage factor $k = 2$ corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3:2008, Guide to the Expression of Uncertainty in Measurement (GUM:1995)

Indicative value

	Equivalent spherical diameter	
	Certified value [2] [nm]	Uncertainty [3] [nm]
Number-based modal diameter [1]	27.8	1.5

1) As obtained by electron microscopy (transmission electron microscopy/scanning electron microscopy).

2) Unweighted mean value of the means of accepted sets of data each set being obtained in a different laboratory and with the method of determination indicated in the respective line of the table. The certified value and its uncertainty are traceable to the International System of Units (SI).

3) The certified uncertainty is the expanded uncertainty with a coverage factor $k = 2$ corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3:2008, Guide to the Expression of Uncertainty in Measurement (GUM:1995)

Additional material information

Measurement method	Information value
ζ-potential by ELS	-48 mV
pH	8.8

1 Table of content

2 Glossary

ACF	Autocorrelation function
ANOVA	Analysis of variance
AUC	Analytical ultracentrifugation
CC	Correlation function, methods of cumulants
CI	Confidence interval
CLS	Centrifugal liquid sedimentation
CONTIN	Algorithm for evaluation of the autocorrelation function by an by an inverse Laplace transformation
CRM	Certified reference material
d_{SAXS}	SAXS particle diameter
DLS	Dynamic light scattering
ELM	Electrophoretic mobility
ELS	Electrophoretic light scattering
EM	Electron microscopy
ERM®	European Reference Materials, CRM trademark owned by the European Commission
FFF	Field flow fractionation
ILC	Interlaboratory comparison
IRMM	Institute for Reference Materials and Measurements, an institute of the European Commission's Joint Research Centre (JRC)
ISO	International Organization for Standardization
JRC	Joint Research Centre
LTS	Long-term stability
$MS_{between}$	Mean square between groups from an ANOVA
MS_{within}	Mean square within groups from an ANOVA
n	Number of replicates
NNLS	Non-negative least squares
p	Number of data sets
pH	Absolute decadic logarithm of the hydronium ion activity in a solution
PI	Polydispersity index
PSD	Particle size distribution
QCM	Quality control material
$r_{Guinier}$	Guinier radius
rel	Index identifying relative standard deviations and uncertainties
SAXS	Small angle X-ray scattering
SEM	Scanning electron microscopy
SI	International System of Units
STS	Short-term stability
s	Standard deviation of dataset means in the characterisation study
s_{bb}	Between- bottle (= ampoule) standard deviation; index rel added as appropriate
$s_{between}$	Standard deviation between-groups (ANOVA); index rel added as appropriate
s_{wb}	Standard deviation within bottle (= ampoule); index rel added as appropriate
s_{within}	Standard deviation within groups (ANOVA); index rel added as appropriate
t	t-factor
TEM	Transmission electron microscopy

u	Standard uncertainty
u^*_{bb}	Between-bottle (=ampoule) heterogeneity that could be hidden by method repeatability; index rel added as appropriate
u_{bb}	Uncertainty related to a possible between-bottle (=ampoule) heterogeneity; index rel added as appropriate
u_{CRM}	Combined uncertainty of the certified value; index rel added as appropriate
u_{char}	Uncertainty of the characterisation; index rel added as appropriate
U_{CRM}	Expanded uncertainty of a certified value; index rel added as appropriate
u_{lts}	Uncertainty of long-term stability; index rel added as appropriate
u_{rec}	Uncertainty estimated from a rectangular distribution; index rel added as appropriate
u_{sts}	Uncertainty of short-term stability; index rel added as appropriate
v/v	volume fraction

3 Introduction

Nanoparticles are particles with external dimensions between 1 nm and 100 nm [1], that may exhibit unique properties due to their size. In order to understand the different properties of nanoparticles, reliable size and size distribution measurements are needed. In this respect, appropriate reference materials including quality control and calibration materials are necessary [2]. A variety of techniques exists to analyse the size and size distribution of nanoparticles in a suspension. Most of these techniques try to describe the size of the particle with one diameter, thereby simplifying the reality, which is that most particles are not perfectly spherical. As a consequence of this, most techniques produce results which are "equivalent spherical" diameters.

Different techniques report different particle diameters due to the different measurement principles used for establishing the equivalent particle diameter. Discrepancy of results obtained with different sizing techniques is expected: particle size is a method-defined measurand [3]. The certified and indicative values of the ERM-FD304 material are specified in this report as equivalent spherical diameters corresponding to the methods used, and to the type of distribution reported. A summary of the techniques used in this study is given below.

1. Dynamic light scattering (DLS) measures the fluctuation of light that is scattered by a quiescent particle suspension. The fluctuation is due to on-going changes of the particle positions by Brownian motion and can be related to the diffusion coefficient of the particles and their hydrodynamic diameter, respectively. Some DLS instruments analyse the intensity fluctuations in the time domain by correlation function analysis , which can be evaluated by the method of cumulants or more sophisticated curve-fitting methods like non-negative least squares (NNLS) or the CONTIN algorithm [4], other DLS instruments analyse the signal fluctuation via frequency analysis [5].
2. The centrifugal liquid sedimentation (CLS) method determines the Stokes diameter of suspended nanoparticles by measuring the velocity of the moving particles during application of a centrifugal force. Depending on their size and density, particles will sediment at different velocities. The velocity of sedimentation decreases with decreasing size for particles of equal density [6]. In the line-start CLS method, a small volume of a dilute suspension of particles is injected into the centre of a spinning disc [7]. The spinning disc chamber is filled with a liquid (e.g. sucrose solution) that has a slight density gradient, so that the liquid at the outside edge of the ring is slightly denser than the one near the inside edge. The local particle concentration at a defined radial position is measured as function of time. From this the distribution of settling velocity or Stokes diameter is derived. In the homogeneous CLS method (also called AUC or Photocentrifuge), the disc is replaced with a rectangular cell or cuvette containing the particle suspension [6]. Here, particles do not pass through a pre-constructed gradient , but sediment out from their native dispersant. Two different measurement and evaluation techniques exist, namely the photocentrifuge and analytical ultracentrifugation. For the photocentrifuge, ISO 13318 [6, 7] is applied, whereas for the analytical ultracentrifuge the particle size distribution is obtained after converting a sedimentation coefficient distribution [8].
3. Scanning electron microscopy (SEM) is a method that uses a beam of electrons, accelerated to high energy and focused on the sample, to image the sample surface. In this study, the EM samples are substrates covered with well-dispersed nanoparticles for particle imaging and analysis. The focused electron beam generates secondary and backscattered electrons and X-rays that will allow one to obtain topographical and chemical information about the sample. In a SEM, the focused electron beam interacts with the sample at the surface whereas in a transmission electron microscopy (TEM), electrons passing through the sample are detected. Particle size distributions are calculated from the images. The SEM/TEM images are analysed using image-analysis-software to evaluate the individual nanoparticle sizes and particle size distributions [9].

EM, be it SEM or TEM differs from the other methods inasmuch as the sample must be dried. This drying process may significantly alter agglomeration status or lead to changes in particle size due to removal of the liquid-particle interface. EM measurements are therefore not necessarily representative for particles in suspension. Nevertheless, EM is a frequently used method and was therefore included in the scope of the certification.

4. In a small angle X-ray scattering (SAXS) experiment the sample of nanoparticle suspension is penetrated by an X-ray beam (transmission mode). The internal structure of the sample (e.g. interfaces between particles and dispersant) causes scattering of X-rays in all directions. The scattering vector is defined in terms of the scattering angle θ and the wavelength λ of the radiation. The scattering curve, meaning intensity as a function of the scattering vector, contains information about the particle shape, size and size distribution [10].
5. In this study, the zeta potential is measured by determining the electrophoretic mobility (ELM) of the particle of interest. ELM is the mobility of the particle under the influence of an applied electric field relative to the liquid in which it is suspended [11].
6. pH was measured by potentiometry, using traditional pH electrodes calibrated with commercial buffer solutions.

The particle sizing methods and the corresponding equivalent particle diameters presented in this certification report are summarised in Table 1.

Table 1: Particle sizing methods and the corresponding equivalent particle diameter as a measurand

Method	**Measurand**
Dynamic Light Scattering (DLS) - Frequency analysis or correlation function analysis with the method of cumulants or other methods	-Scattering intensity-weighted harmonic equivalent spherical mean diameter - Volume-weighted harmonic equivalent spherical mean diameter
Centrifugal Liquid Sedimentation (CLS) - line-start method	- Extinction intensity based equivalent sphericamodal Stokes diameter - Volume based modal Stokes equivalent spherical diameter
Transmission Electron Microscopy (TEM) / Scanning Electron Microscopy (SEM)	- Number based modal spherical diameter
Small Angle X-ray Scattering (SAXS)	- Scattering intensity-weighted mean equivalent spherical diameter - Volume-weighted mean equivalent spherical diameter

4 Participants

4.1 Project management and evaluation

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE

4.2 Processing

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE

Grace Davison GmbH, Worms, DE

4.3 Homogeneity study

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE

4.4 Stability study

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE

4.5 Characterisation

Participants are listed in alphabetical order. Accreditation body and certificate number are stated for laboratories with ISO/IEC 17025 [16o] accreditation for the measurements in question.

Agfa-Gevaert NV, Research and Development Materials, Mortsel, BE

Anton-Paar GmbH, Graz, AT

BASF SE, Polymer Physics, Ludwigshafen, DE

Beijing Center for Physical and Chemical Analysis BCPCA, Beijing, CN,
(accreditation to ISO/IEC 17025, China National Accreditation Service, L0066 2002-10, GB/T 19627-2005/ISO13321-1996: Particle size analysis-Photon Correlation Spectroscopy)

Capsulution Pharma AG, Berlin, DE

Federal Institute for Materials Research and Testing BAM I.3 "Structure Analysis; Polymer Analysis", Berlin, DE

Flemish Institute for Technological Research VITO, Materials Technology, Mol, BE

Horiba Instruments Inc., Irvine, US

Industrial Technology Research Institute ITRI, Hsinchu, TW,
(CMS/ITRI, accreditation to ISO/IEC 17025, Taiwan Accreditation Foundation, N0688/2000.10.15. NTRC/ITRI, accreditation to ISO/IEC 17025, Taiwan Accreditation Foundation, 1569/2006.8.10.)

European Commission, Joint Research Centre, Institute for Health and Consumer Protection, Ispra, IT

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Reference Materials Unit, Geel, BE

LUM GmbH, Berlin, DE

Malvern Instruments Ltd., Malvern, UK

Malvern Instruments Inc., Westborough, US

Max Planck Institute of Colloids and Interfaces, Potsdam-Golm, DE

microParticles GmbH, Berlin, DE

MVA Scientific Consultants, Duluth, US
(accredited to ISO/IEC 17025, American Association for Laboratory Accreditation, 2096.01)

National Center for Nanoscience and Technology, Institute of High Energy Physics, CAS Key Lab for Biological Effects of Nanomaterials and Nanosafety, Beijing, CN

Nanotechnology Characterisation Laboratory, Frederick, US

National Measurement Institute Australia NMIA, Lindfield, AU

National Physical Laboratory (NPL), Materials Division, Teddington, UK

National Institute of Standards and Technology (NIST), Gaithersburg, US

DANNALAB B.V., Enschede, NL

Particle Metrix GmbH, Meerbusch, DE

Philips Research-MiPlaza, Eindhoven, NL

Physical-Technical Federal Institution (Physikalisch-Technische Bundesanstalt PTB), Berlin, DE

Rigaku Innovative Technologies Inc., Auburn Hills, US

RIKILT, Institute of Food Safety, Wageningen, NL

Sympatec GmbH, Clausthal-Zellerfeld, DE

SIRRIS, Seraing, BE

Technical University Bergakademie Freiberg, Institute MVT/AT, Freiberg, DE

Technical University of Denmark, the National Food Institute, Soborg, DK

Technical University of Dresden, Institute of Process Engineering and Environmental Technology, Dresden, DE

University of Namur-FUNDP, Nanotoxicology Platform characterisation Group, Namur, BE

University College Dublin, Dublin, IE

5 Material processing and process control

The starting material chosen for the production of ERM-FD304 is colloidal silica called Ludox® TM-50 produced by Grace Davidson, Worms, DE. Information about the raw material is summarised in Table 2.

Table 2: Information about the Ludox® TM-50 raw material

Property	**Value**	**Information source**
Raw material	Colloidal silica	Manufacturer
Nominal particle size	30 nm to 32 nm	Manufacturer
pH, (25 °C)	8.9 to 9.4	Manufacturer
Viscosity (25 °C)	40 mPa·s	Manufacturer
Mass fraction of silica	50 %	Manufacturer
Particle density [g/cm^3]	2.2	Manufacturer
Zeta potential	-37 mV to -39 mV	Manufacturer
Composition of suspension	pH at 25 °C: 9 Specific surface area: 140 m^2/g titrable alkali as Na_2O: 2 g/kg Chlorides as NaCl: 0.3 g/kg Sulfates as Na_2SO_4: 0.8 g/kg	Manufacturer
Particle morphology	nearly spherical	TEM measurements commissioned by IRMM

A TEM image of the raw material Ludox® TM-50 particles, obtained by an independent laboratory by drying a drop of a diluted material (dilution with ultrapure water to 0.5 % (m/m)) on a TEM - grid is shown in Figure 1. The number-weighted particle size distribution was monomodal with a modal diameter of about 25 nm. Some larger and smaller particles are visible between the bulk of the regular sized particles, as shown in Figure 1.

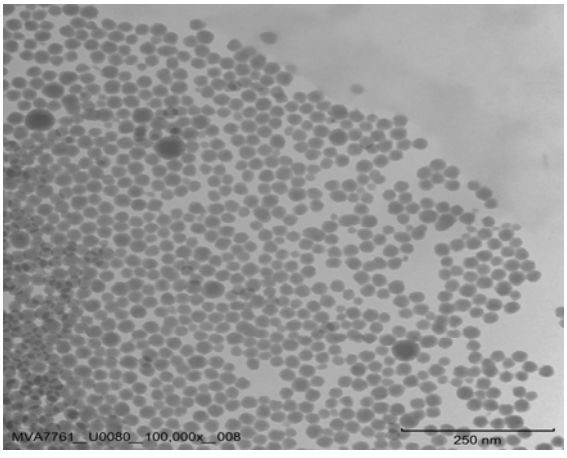

Figure 1: TEM micrograph of dried Ludox® TM-50. Measurements were not performed by the manufacturer.

Dilution of the 50 % suspension and ampouling was performed at IRMM. The target silica mass fraction for ERM-FD304 was 0.5 % (m/m). 25 L of a 0.5 % (m/m) silica suspension was prepared by dilution of 227.0 g Ludox® TM-50 with 23553.6 g deionised water (resistivity 18.2 MΩ · cm, Millipore SAS, Molsheim, FR) in a clean plastic tank, mixed by hand-shaking. The

sample was left standing overnight to remove large particles by sedimentation. On the next day, the suspension was ampouled withut stirring, to avoid re-suspending sedimented particles.

Pre-scored amber glass ampoules (obtained from Nederlandse Ampullenfabriek, NL) of 10 mL were chosen for the processing of ERM-FD304. Before filling, the glass ampoules were opened, rinsed with ultrapure water and dried in an oven. The ampoules were filled with approximately 9.5 mL of colloidal silica using a manually operated dispenser, and flame sealed. A total of 2146 ampoules of ERM-FD304 were produced. Each ampoule was labelled with the batch code (ERM-FD304) and an individual identification number.

Measurement of the silica mass fraction by oven drying at 105 °C showed that the particle mass fraction of ampoules 1-500 and 1811-2146 differed from the particle mass fraction of ampoules 501-1810. Therefore, the ampoules at the beginning and at the end of the filling sequence were discarded and only samples 501 to 1810 were retained for distribution. Therefore, the notion "the whole batch" in this document refers only to samples number 501 to 1810.

6 Assessment of homogeneity

A key requirement for any reference material is the equivalence between the various units. In this respect, it is not relevant whether the variation between units is significant compared to the analytical variation, but whether this variation is significant to the certified uncertainty. Consequently, ISO Guide 34 requires RM producers to quantify the between unit variation. This aspect is covered in between-unit homogeneity studies.

The within-unit heterogeneity does not influence the uncertainty of the certified value when the minimum sample intake is respected, but determines the minimum size of an aliquot that is representative for the whole unit. Quantification of within-unit heterogeneity is therefore necessary to determine the minimum sample intake.

During the production of ERM-FD304, different methods such as DLS, CLS, SAXS, SEM and TEM were used in the homogeneity, stability and characterisation studies. Even if the measurand is method-defined, they all measure the same particles. Assessment of homogeneity only requires demonstration that the content of each ampoule is the same. Therefore, one method is sufficient to confirm homogeneity. If the methods have different precision, then the method with the highest precision should be chosen. Among the tested methods, DLS has the best repeatability; therefore the main assessment was based on DLS. In addition, CLS measurements were chosen to confirm the DLS results. Moreover, CLS has the advantage over DLS that it has a much higher sensitivity to detect multimodal distributions than DLS.

6.1 Between-unit homogeneity

Between-bottle homogeneity was tested using DLS and CLS. For the CLS study, 6 samples were selected using a random stratified sampling scheme, thus ensuring that the complete batch was covered. The intensity-based modal Stokes diameter was determined on a DC20000 Disc CentrifugeTM (CPS Instruments Inc., Stuart, Florida, US). Two subsamples per ampoule were measured under repeatability conditions by CLS.

For the homogeneity testing by DLS, 18 samples were selected using a random stratified sampling scheme and were tested on a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). Two replicate determinations per ampoule were performed under repeatability conditions. Triplicate analyses were performed per subsample. The measurand of this DLS analysis test was the z-average particle size with the method of cumulants, as obtained from the analysis of the. autocorrelation function

The measurement scheme is shown in Figure 2. Sample intakes were 2.5 mL for DLS and 0.2 mL for CLS.

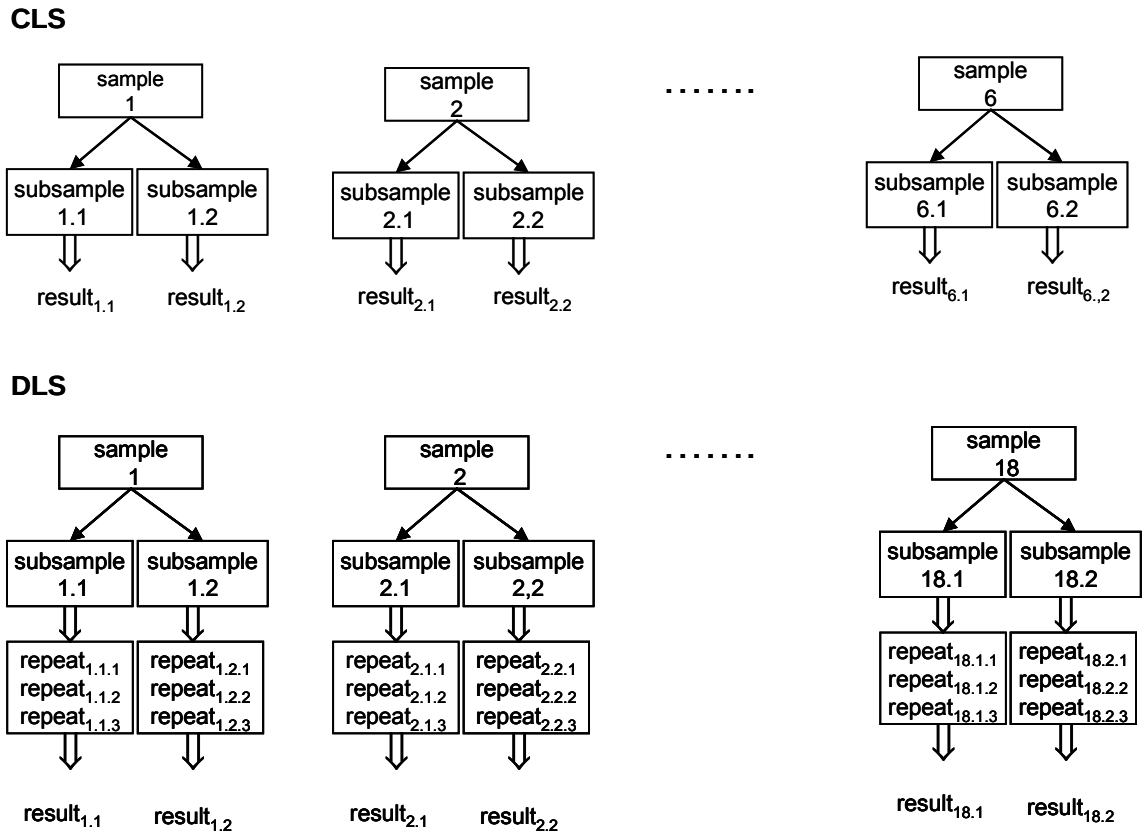

Figure 2: Measurements scheme of the homogeneity study

Grubbs-tests at 99 % confidence levels were performed to detect potentially outlying individual results as well as outlying ampoule averages. Regression analyses were performed to evaluate potential trends in the analytical sequence as well as trends in the filling sequence. It was furthermore checked whether the individual data and ampoule averages follow normal distributions using normal probability plots and whether the individual data are unimodally distributed using histograms.

The results of these evaluations are shown in Table 3. The results of the measurements themselves are depicted in Annex A.

Table 3: Descriptive evaluation of the ERM-FD304 results for each method. DLS measurand is the intensity-weighted harmonic mean particle diameter and the line-start CLS measurand is the intensity-based modal Stokes particle diameter

Method	Trends		Outliers		Distribution	
	Analytical sequence	Filling sequence	Individual results	Bottle means	Individual results	Bottle means
DLS	no	yes	none	none	normal	normal
CLS	no	no	1	none	not normal (outlier)	normal

Regression analyses were performed to evaluate potential trends in the analytical sequence as well as trends in the filling sequence. No trends were detected for the CLS data. One outlying datapoint was found in the CLS data, which was retained, as no technical reason for the exclusion was found. Individual values and unit means follow normal distributions.

The DLS data, however, showed a small (0.3 pm/ampoule or 0.4 nm over the whole batch) but significant (99 % confidence level) slope in the filling sequence. The slope is small

enough to make the material useful as reference material. This is also shown by the fact that individual values as well as unit means follow normal distributions.

Results obtained by CLS and DLS were evaluated using single-factor ANOVA. For DLS, The evaluation was based on the mean of the three repeat repeats per subsample. Standard deviations within bottles (s_{wb}) and between bottles (s_{bb}) as well as u^*_{bb}, the maximum heterogeneity that could be hidden by method repeatability, were calculated as shown in equation 3 [12]. u^*_{bb} is comparable to the limit of detection of an analytical method, yielding the maximum heterogeneity that might be undetected by the given study setup.

Method repeatability ($s_{wb,rel}$), between–unit standard deviation ($s_{bb,rel}$) and $u^*_{bb,rel}$ were calculated as shown in equations 1 and 2.

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\bar{y}} \quad (1)$$

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}} \quad (2)$$

$$u^*_{bb,rel} = \frac{\sqrt{\frac{MS_{within}}{n}} \sqrt[4]{\frac{2}{v_{MSwithin}}}}{\bar{y}} \quad (3)$$

MS_{within} mean square within a unit from an ANOVA

$MS_{between}$: mean squares between-unit from an ANOVA

$\bar{y}$ average of all results of the homogeneity study

n: average number of replicates per unit

$v_{MSwithin}$: degrees of freedom of MS_{within}

The results of these evaluations are shown in Table 4. As the data obtained by DLS showed a trend in the filling sequence, a second evaluation was applied. Here, u_{rec} was estimated using a rectangular distribution between the highest and lowest unit average. The corrected uncertainty in those cases where there was a significant trend in the filling sequence is given in equation 4:

$$u_{rec} = \frac{|highest\ result - lowest\ result|}{2 \cdot \sqrt{3} \cdot (\bar{y})} \quad (4)$$

Table 4: Results of the homogeneity study for ERM-FD304.
n.c.= cannot be calculated as $MS_{between} < MS_{within}$

Number	Method	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]	$u_{rec,\ rel}$ [%]
1	CLS	2.52	n.c.	1.35	
2	DLS	0.255	0.322	0.104	0.409

As expected, the better repeatability of DLS compared to CLS results in narrower boundaries for potential heterogeneity. Using ANOVA, which assumes the unit means to follow a normal

distribution, a between-unit heterogeneity of 0.32 % was estimated, which is virtually the same as the between-unit heterogeneity obtained from the rectangular model (0.41 %). Due to the higher repeatability standard deviation, CLS could not quantify this slight heterogeneity but the upper limit for between-unit heterogeneity ($u^*_{bb,rel}$; 1.35 %) agrees with the values obtained by DLS. Therefore, 1.35 % and 0.409 %, respectively, were adopted as uncertainty contributions for CLS and DLS.

6.2 Within-unit homogeneity and minimum sample intake

Within-bottle (= within ampoule) heterogeneity is closely related to the minimum sample intake. Due to the intrinsic heterogeneity, individual subsamples of a material will not contain the same number and type of particles; hence the mean/modal diameter will change. The smallest subsample that is representative for the complete ampoule is the minimum sample intake. The larger the intrinsic heterogeneity, the larger the minimum sample intake will be.
Minimum sample intake for the different methods was determined from the characterisation study. The sample intake that still yielded results with acceptable repeatability to be included in the respective studies was taken as minimum sample intake. The lowest sample intake for CLS in the characterisation study was 170 μL, for DLS 100 μL and for EM 3 μL (see Annex C).
To simplify the certificate, similar minimum sample intakes for different methods are desirable. Therefore, the minimum sample intake for CLS and DLS is given as 170 μL.
The minimum sample intake for the EM is 3 μL. In any case, enough sample should be taken to assure measuring of minimum 500 particles on the testing stub/grid.

7 Stability

Stability testing is necessary to establish conditions for transport to the customer as well as conditions for storage. Due to the dilution process, particles might agglomerate, or dissolve, even if they were prepared from an initially stable suspension. Time and temperature were regarded as the most relevant influences on the stability of the materials. Therefore, only the influences of time and temperature were investigated.

The stability studies were conducted as isochronous stability studies [13] and classical stability studies. In the former type, samples are stored for a certain time interval under the test conditions. After that time, samples are moved to conditions where further degradation is negligible ("reference condition"), effectively "freezing" the degradation status of the materials. This setup allows analysis of materials of various exposure times under repeatability conditions, thus greatly improving the sensitivity of the study to detect degradation. The storage design for the isochronous short-term stability (STS) study is shown in Figure 3.

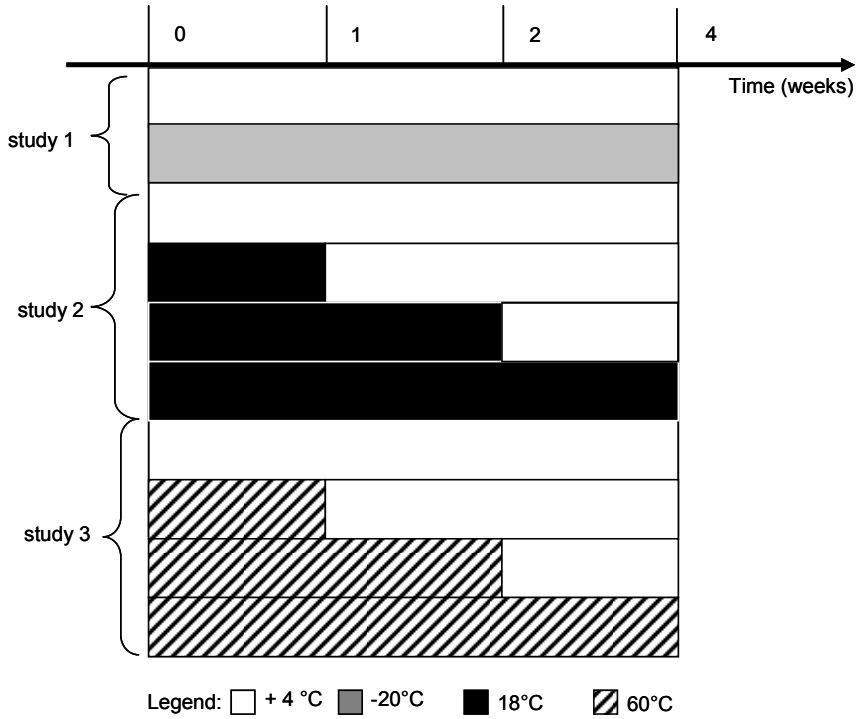

Figure 3: Storage scheme for the isochronous measurements – short-term study

In case of the "classical studies", samples stored at +18 °C, were measured at different times. This study has the disadvantage that day-to-day- analytical variation is confounded with potential material degradation, but this drawback was made up by the longer duration of this study.

7.1 Short-term stability study

Samples for the short-term study were selected from the whole batch following a random stratified sampling scheme and were analysed under repeatability conditions. Samples were stored for up to 4 weeks at -20 °C, +18 °C and +60 °C, respectively. Tests at -20 °C were carried out as a confirmation study since previous experience with the same material has revealed irreversible sedimentation when the sample is frozen. Tests at 60 °C were carried out to monitor changes at extreme condition which can occur during sample transport. Storage at 4 °C was defined as the reference condition for the short-term stability study. DLS measurements evaluated by the method of cumulants and measurements of the ζ-potential were performed on a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). Particle sizes were also determined by CLS on a DC20000 Disc CentrifugeTM (CPS Instruments Inc., Stuart, Florida, US). In addition, the pH value was measured. The detailed setups of the studies were as follows:

- DLS, ELS, pH, 60 °C: 2 ampoules each stored for 0, 1, 2 and 4 weeks. Two subsamples per ampoule were tested, with triplicate instrument readings per subsample.
- CLS, 60 °C: 1 ampoule each stored for 0, 1, 2 and 4 weeks. Two subsamples per ampoule were tested for each time.
- DLS, ELS, pH, 18 °C: 4 ampoules each stored for 0, 1, 2 and 4 weeks. Two subsamples per ampoule were tested, with triplicate instrument readings per subsample.
- DLS, -20 °C: 2 ampoules were stored for 4 weeks

The studies were evaluated individually for each instrument and temperature. Results were screened for outliers using a Grubbs test, but outliers would only be excluded if a technical reason could be identified. Linear regressions were performed and the slopes were tested for

significance at 99 % confidence levels. The results of these evaluations are summarised in Table 5, whereas graphical representations of the studies are given in Annex B.

Table 5: Results of the evaluation of the short-term stability study. Outliers and slopes were tested on a 99 % confidence level.

	18 °C		60 °C	
Method	Number of individual outlying results	Significance of the trend on a 99% confidence level	Number of individual outlying results	Significance of the trend on a 99% confidence level
DLS_{CC}	none	no	none	no
CLS			none	no
ELS	none	no	none	no
pH	none	no	1	no

No outliers were detected in the 18 °C study and none of the slopes was significantly different from zero. For the 60 °C study, only one outlying value for pH was detected, the removal of which did not influence the significance of the slopes. The linear regression showed significant slopes on a 95 %, but not 99 % confidence level for DLS and ELS. However, this slope is technically negligible.

7.2 Long-term stability study

Long-term stability was established in a classical stability study over a time period of 3 years of storage at 18 °C using two different DLS instruments as well as CLS. Data have been analysed according to ISO 22412 [17]obtaining an intensity-weighted harmonic mean by either ACF analysis with the method of cumulants as described in ISO 13321 [18] (DLS_{cc}) or frequency analysis (DLS_{FA}). The studies for the different methods were of different lengths: DLS_{FA} lasted for 36 months, DLS_{CC} for 15 months and CLS for 30 months.

The studies were evaluated individually for each instrument. Results were screened for outliers using a Grubbs test, but only outliers that were dubious on technical grounds were excluded. Linear regressions were performed and the slopes were tested for significance at 99 % confidence levels. The results of these evaluations are summarised in Table 6, whereas graphical representations of the studies are given in Annex B.

Table 6: Results of the evaluation of the long-term stability study. Outliers and slopes were tested on a 99 % confidence level.

Method	Number of individual outlying results	Significance of the trend on a 99% confidence level
DLS_{FA}	none	no
DLS_{CC}	none	no
CLS	none	no

No significant slopes or outlying results were obtained on the long-term stability study (LTS) at 18 °C using DLS and CLS method.

As expected, freezing of the samples led to irreversible agglomeration with particle diameters up to 3 μm.

7.3 Estimation of uncertainties

Uncertainties of stability during dispatch and storage were estimated as described in [14] for each method. For this approach, the uncertainty of the linear regression line with a slope of zero is calculated. The uncertainty contribution is then estimated as a chosen shelf life

multiplied with the uncertainty of the regression lines. The following uncertainties were estimated:

- u_{sts}, the uncertainty of the particle size due to dispatch. This was estimated from the 60 °C studies for a time of one week. The uncertainty therefore describes the possible change during a dispatch at 60 °C lasting for one week.
- u_{lts}, the uncertainty contribution for DLS was calculated to describe possible degradation for 24 months at 18 °C.

The results of these evaluations are summarised in Table 7.

Table 7: Uncertainties of stability during storage and dispatch.
$u_{sts,rel}$ was calculated for a temperature of 60 °C and 1 week; $u_{lts,rel}$ was calculated for a storage temperature of 18 °C and 24 months

Method	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]
DLS_{CC}	0.072	0.445
DLS_{FA}	not tested	1.24
CLS	0.56	3.93
ELS	1.0	not tested
pH	0.30	not tested

Uncertainties of stability during transport are very low for particle size, ranging from 0.07 % for DLS to 0.56 % for CLS. If the slope for DLS, that is significant on a 95 % confidence level, is included, the uncertainty is higher (0.13 %), but still very low, showing that the decision to discard the slope is justified.

Despite the shorter duration, the lower variation of results obtained by the method of cumulants results in a lower uncertainty than obtained for the frequency analysis. Because of the worse repeatability, a significantly larger uncertainty of stability was obtained for the CLS method.

After the certification campaign, the material will be subjected to IRMM's regular stability monitoring programme to demonstrate its further stability.

8 Characterisation

The material characterisation was based on an intercomparison of expert laboratories, i.e. the properties of the material were determined in different laboratories to demonstrate the absence of a measurement bias. This approach aims at randomisation of laboratory bias, which reduces the combined uncertainty.

8.1 Selection of participants

Participants for the characterisation study were selected based on technical as well as quality management criteria.

Before the characterisation study, participants had to demonstrate their technical competence and proficiency in sizing of nanoparticles by participation in an interlaboratory comparison (ILC) organised by IRMM [15] or in at least one other ILC of their choice. In addition, laboratories had to demonstrate that measurements were made in compliance with ISO/IEC 17025 [16], although formal accreditation was not required.

Fulfilment of the quality management requirements ensured that the technical standard was maintained from the time of demonstration in the ILC to the actual measurement. Few participating laboratories are accredited to ISO/IEC 17025 [16]. Where measurements are covered by the scope of accreditation, the accreditation number is stated in the list of participants (Section 2). Assessment of compliance with ISO 17025 was based on self-

declaration using a questionnaire, in which they confirmed that with regard to training, method validation, method documentation and authorisation, and instrument maintenance, the requirements of ISO/IEC 17025 were met.

The scatter of the results for the ζ-potential determination in the ILC organised by IRMM was too large to allow a positive demonstration of proficiency. Therefore, as competence could not be demonstrated on beforehand, it was clear that the zeta potential value obtained from the characterisation study could not result in a certified value.

8.2 Study setup

The aim of the study was the characterisation of the colloidal silica candidate reference material via an interlaboratory study with subsequent technical evaluation. Basis of the certification approach is the randomisation of the (unknown) laboratory biases. Randomisation is only successful if,

1) the measurements within one laboratory are performed under reproducibility conditions, and
2) if results from different laboratories are indeed independent.

In this respect, the participating laboratories were asked to strictly follow the test protocol sent together with the samples. This protocol included tests on multiple samples of ERM-FD304 on three different days as well as tests on a Quality Control Material (QCM), and required the registration and reporting of all the requested test details into a specifically prepared test report form. Both documents, the test protocol and the test report form were prepared for each of the five methods used in this characterisation study. The instruments and the methods used are summarised in Annex C. The information in this annex is presented as reported by the participants.

The participants received three ampoules of ERM-FD304 and one QCM sample. In total, two independent results (= from independent subsamples or 'aliquots') per ampoule (ERM-FD304) had to be measured.

On the first day, the measurements on 2 aliquots of ERM-FD304 and 2 aliquots of the QCM had to be performed. Each aliquot had to be measured in triplicate (= depending on the instrument set up preferentially three instrument readings) under repeatability conditions. This measurement schedule which had to be strictly followed by the participants is shown in Table 8. It was requested to measure the suspension in the ampoules as received and, if possible, not to do any sample pre-treatment prior to the measurements (i.e. without filtration, dilution, centrifugation, sonication).

Table 8: Overview of the measurements to be performed on the 3 different days

Measurement sequence per day	Day 1	Day 2	Day 3
1.	QCM aliquot 1	ERM-FD304 aliquot 1 ampoule 2	ERM-FD304 aliquot 1 ampoule 3
2.	ERM-FD304, aliquot 1 ampoule 1	ERM-FD304 aliquot 2 ampoule 2	ERM-FD304 aliquot 2 ampoule 3
3.	ERM-FD304 aliquot 2 ampoule 1	-	-
4.	QCM aliquot 2	-	-

The participating laboratories were also requested to give estimations of the expanded uncertainties of the mean value for each ampoule. No approach for the estimation was prescribed, i.e. top-down and bottom-up uncertainty budgets were regarded as equally valid procedures.

8.2.1 DLS

The characterisation of the colloidal silica candidate CRM ERM-FD304 by the DLS method was performed in terms of the intensity-weighted harmonic mean size of the particle size distribution, in general accordance with the standards ISO 22412 [17] and ISO 13321 [18]. Participants were instructed to use a refractive index of 1.46 [19]. for silica and 1.33 for the medium (water).

A blind colloidal silica sample with a nominal particle mass fraction of 0.75 % was sent together with the samples as QCM. The QCM actually corresponded with the colloidal silica used for the ILC mentioned earlier [15], which was shown to have a mean diameter (DLS, method of cumulants of ACF analysis) of nominally 36 nm.

8.2.2 CLS

The measurand of the CLS method was the intensity-based modal Stokes particle diameter corresponding with the main mode in the particle size distribution of the colloidal silica candidate reference material, in general accordance with the standard test methods ISO 13318-1:2001 [6] and ISO 13318-2:2007 [7]. This includes both the line-start method and homogeneous techniques, implemented in disc centrifuges or cuvette centrifuges. The same QCM as for the DLS was also used in this case.

Participants were instructed to use a particle density of 2.305 g/cm^3 for the evaluation of their measurements [19].

8.2.3 EM (SEM/TEM)

The characterisation of ERM-FD304 by EM in terms of the modal diameter of the main mode in their particle size distribution was based on the analysis of electron microscopy images in general accordance with the standard static image analysis method ISO 13322-1:2004 [20]. This includes both the scanning electron microscopy (SEM) and transmission electron microscopy (TEM) techniques.

The same QCM as for the DLS was also used in this case.

8.2.4 SAXS

ERM-FD304 was characterised in terms of its particle size using Small Angle X-ray Scattering (SAXS) [10].

For this technique, a QCM consisting of colloidal gold was sent with the sample, namely RM 8012 (NIST, Gaithersburg, US; [21]) This material consists of a suspension of colloidal Au with a nominal particle concentration of 48 µg/g and SAXS particle diameter of 24.9 ± 1.2 nm.

8.2.5 ELS and pH

ERM-FD304 was characterised in terms of pH and zeta potential, the latter via electrophoretic mobility measurements. Carbon dioxide from air reacts with water and as a result, changes in zeta potential are to be expected after opening an ampoule due to the changing of pH. Therefore, it was explicitly mentioned that immediately after opening an ampoule, two independent aliquots had to be prepared, protected from air, and tests had to be performed as soon as possible.

Polystyrene latex microspheres dispersed in an aqueous buffer (pH 9.2) and packed in a 10 mL syringe (Zeta Potential Transfer Standard, assigned zeta-potential value = -68 ± 6.8 mV, Malvern Instruments Ltd., UK) were used as QCM for ELS.

8.3 Technical evaluation

35 laboratories took part in the characterisation study, most of them offering several different methods (see Annex C) and measurement results. The geographical distribution of the participants was as follows: 6 from America, 3 from Asia, 25 from Europe and 1 from Australia. The information on measurement parameters in Annex C shows that participants used different numbers for viscosity etc, which certainly also contributed to between-laboratory variation.

Before starting the technical evaluation, it was checked if the results of the QCMs provided for each method agreed with the assigned values in the NIST report of investigation [21] for the SAXS method, the Malvern certificate for the ELS method and the mean value of the data from the IRMM ILC study [15].

The instruments used for all the methods are summarised in Annex C. The results of the studies are depicted in Annex D.

8.3.1 Polydispersity

The initial characterisation by TEM had already shown that ERM-FD304 is to a certain degree polydisperse. This finding was confirmed during the characterisation study (see below):

- Polydispersity indices calculated from DLS (cumulants method) were larger for ERM-FD304 than for ERM-FD100, when comparing the results from the same laboratory.
- One laboratory reported a bimodal distribution by applying SAXS at very small scattering vectors, as discussed in section 8.3.5.
- Field flow fractionation (FFF) coupled to DLS also gives a bimodal particle size distribution as shown in Figure 4. In this technique, particles are fractionated according to their diameter. Larger particles arrive earlier at the detector than smaller ones, hence the time axis (x-axis) is correlated to size (and can be calibrated to size). In this case, DLS was used as detector, which gives size information at each time measured. This fractogram clearly shows that particles in FD304 do not follow a narrow, monodisperse distribution, but the size of particles ranges from about 10 to 40 nm (size scale not shown).

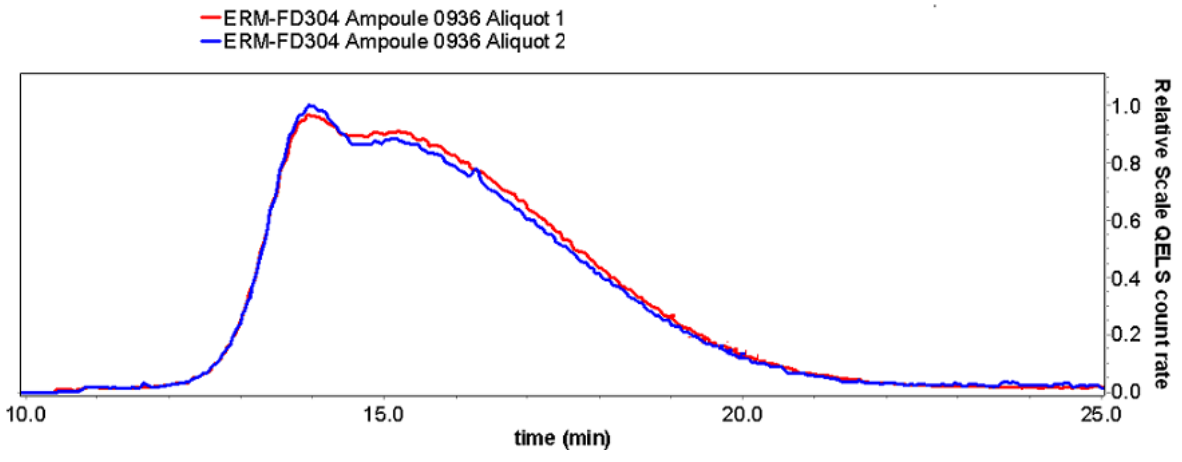

Figure 4: FFF-DLS for ERM-FD304. The y-axis is the count rate of the DLS used as detector.

- The particle size distribution as obtained by TEM (see Figure 5) show a monomodal, but somewhat broad distribution.

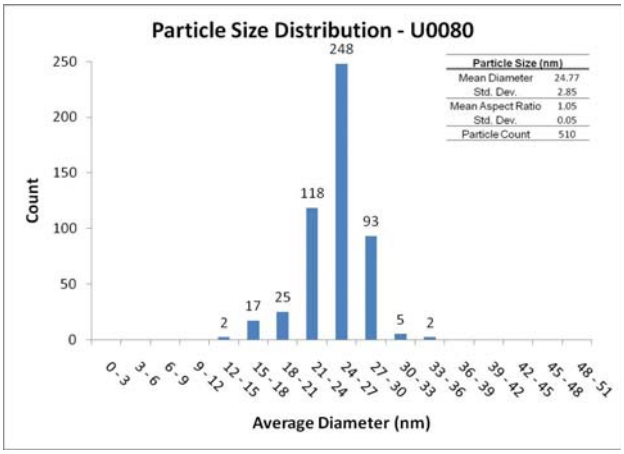

Figure 5: Particle size distribution as obtained by TEM

This polydispersity highlights differences in evaluation algorithms and instrument setups, that are not visible for monodisperse materials with narrow distributions. Therefore, more problems were expected for the evaluation of ERM-FD304 than for ERM-FD100 [22].

Polydispersity increases between-laboratory variation, due to its effects on the different data analysis algorithms of the various DLS methods. ISO 22412:2008 [17] describes different data analysis methods for (cross-) correlation function analysis and describes the principles of frequency analysis. These different methods will deliver similar results if the material is monodisperse, but not necessarily when the material is polydisperse. The most robust method is the correlation function analysis with the method of cumulants, in which the experimental data are fitted to a polynomial series of the intensity correlation function, truncated after the quadratic term, expanded around the average value. As a result, the signal contribution from particles with different diameters is suppressed, resulting in a robust method with a high repeatability. On the other hand the frequency analysis method, or other correlation function analysis methods take account of all information in the correlation function or in the power spectrum, which gives a higher sensitivity to slight changes in the distribution function.

The evaluation of the characterisation study illustrates exactly this. Within one laboratory, the method of cumulants gives consistent, repeatable results, and these results are very comparable to measurements in other laboratories, with other instruments, if they are also using the method of cumulants. On the other hand, instruments calculating a power spectrum with frequency analysis, or analysing the correlation function with, for example, the non-negative least squares (NNLS method), show a wider spread of data, and an average value which is not necessarily comparable to data pools from instruments using the method of cumulants.

8.3.2 DLS

In total, 21 laboratories submitted 24 datasets for DLS using correlation function analysis (evaluated using cumulants analysis or other algorithms) as well as frequency analysis, with reported diameters ranging from 41 to 51 nm. Figure 6 shows all intensity weighted results from the various evaluation methods.

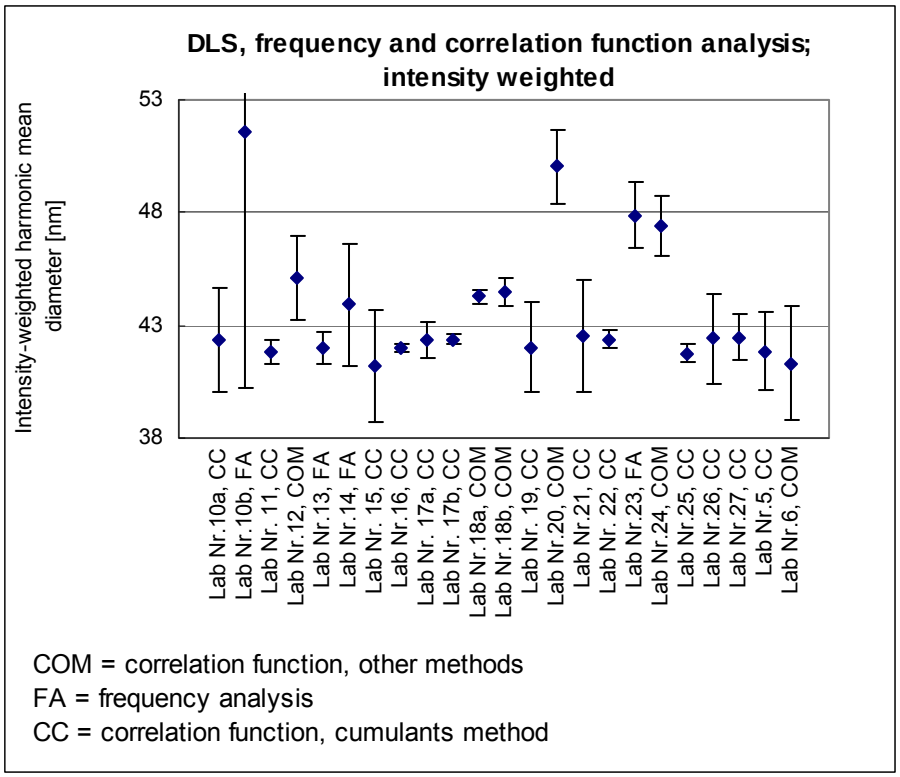

Figure 6: Summary of all DLS results. Error bars are expanded uncertainties as reported by the participants

Evaluation was made even more difficult by the fact that some laboratories reported the evaluation algorithm incorrectly. Several laboratories stated evaluation by the method of cumulants, whereas the raw data kindly provided showed that in fact other algorithms had been used. These results were therefore not included in the set of cumulants data. The methods listed in Annex C list the method information after these corrections.

As has been already observed for ERM-FD100, results from frequency analysis scattered more than those obtained by the method of cumulants [22]. Presumably due to the polydisperse nature of ERM-FD304, results from cumulants analysis and other evaluation techniques for correlation analysis also do not agree. It was therefore decided to limit the evaluation to intensity-weighted particle diameters obtained by cumulants analysis.

The results from laboratory 37 were excluded from the evaluation, since the method (FFF coupled with DLS) was not compatible with the cumulants data from non-fractionated samples.

As the results are method dependent, one could envisage assigning values for results obtained from frequency analysis or from correlation analysis using other evaluation techniques than cumulants. However, the results from frequency analysis scatter too much to make this feasible. Data of non-cumulants correlation analysis agree better, but these algorithms are very instrument specific, making value assignment impossible for this study.

Results for volume weighted diameter obtained by DLS varied significantly more than the intensity weighted data (see Annex D), with three clusters ranging from 27 nm to 44 nm. The results depend strongly on the evaluation method, which is subject to further standardisation efforts. These data were therefore not evaluated any further.

8.3.3 CLS

Ten participants submitted in total ten independent data sets of which six were obtained by line-start disc centrifugal liquid sedimentation (also called differential centrifugal

sedimentation), and 4 by homogeneous centrifugal liquid sedimentation (also called analytical ultracentrifugation (AUC) or photocentrifuge). From the 4 laboratories performing homogeneous CLS, all reported volume based modal diameter and 3 reported intensity based modal particle diameter additionally.

From the 4 data sets obtained by homogeneous CLS, 2 were measured with analytical ultracentrifugation and the other two with photocentrifuge. Three laboratories reported results from the homogeneous CLS method of 22 nm, 26 nm and 42 nm for the intensity based particle diameter whereas the 6 data sets for the line-start method were all close to the mean value of 32 nm.

Data from the homogeneous CLS method differed significantly from the line-start method in terms of the intensity based modal Stokes diameter. It was decided not to pool the data sets of the two different methods together and only further evaluate data originating from the line start method due to insufficient number of results for the homogeneous CLS method.

For the volume based particle diameter, the results form two clusters, with diameters around 25 nm and 31 nm, respectively (see Annex D). As there was no agreement on a common value, no further evaluation was performed.

8.3.4 EM (SEM/TEM)

Eight participants submitted in total eleven independent data sets of which eight were TEM results and three SEM. Laboratory 3, 11 and 15 delivered results both for TEM and SEM. Each laboratory performed the analysis of at least 500 randomly selected and counted particles. No results were discarded for too large deviation from the assigned values of the QCM. The following observations or comments were made:

- TEM laboratory 16 stated that all as-received material samples were diluted to achieve a target mass fraction of 0.075 µL in order to produce a good coverage of particles on the grids.
- Laboratory 11 diluted 300 µL sample with 700 µL ultrapure water, followed by filtration with 0.1 µm filter size for SEM. It applied a dilution of 1:9 by volume and additional filtration with 0.1 µm filter size for TEM measurements.
- SEM laboratory 15 reported a dilution of 1:50 (v/v).

All dilutions were performed with ultrapure water.

8.3.5 SAXS

Five participants submitted one data set each. One laboratory only reported the intensity weighted, but not the volume-weighted particle size distribution. The results of all laboratories agreed with the assigned values of the QCM, so all results were accepted.

Participants used their instrument-specific evaluation techniques. Some of them used the intensity-weighted radius of gyration, whereas others reported a size distribution based on a model function. The participants reported the intensity and volume-weighted Guinier radius for intensity and volume-weighted results. The SAXS particle diameter d_{SAXS} was obtained from the Guiner radius $r_{Guinier}$ via equation 5 [17]:

$$d_{SAXS} = 2\sqrt{\frac{5}{3}} \cdot r_{Guinier} \qquad (5)$$

For both volume and intensity weighted diameter the values were around 26 or 27 nm respectively.

4 laboratories submitted radii of gyration of about 10 nm to 11 nm (particle size diameter of 27 nm to 29 nm), laboratory 31 however reported significantly higher radii of gyration. The

laboratory also reported that the curve consists of two parts, through which a straight line can be fitted. These two different lines then correspond to different particle sizes, which indicates the present of some larger agglomerates. This is consistent with the findings of laboratory 20 and 25 (DLS method) and 37 (FFF with DLS method) which also mentioned the polydisperse nature of the material. Discussion with the other participants using SAXS did not give a clear reason, why this effect did not occur for the other instruments: Possible explanations were different scattering vectors used as well as differences in the evaluation software. The various evaluation approaches used by the participants agreed all individually well with the measured data. It therefore seems that different setups of SAXS instruments and evaluation algorithms lead to different values for ERM-FD304, which excludes assigning a common value that would be valid for all SAXS instruments.

8.3.6 ELS and pH

Ten participants submitted in total ten independent ELS data sets. One result for ζ-potential was excluded, because the results on the quality control sample differed too much from the target value. No effect of the scattering angle used for the measurements on the zeta potential results was noticed.

Ten laboratories submitted results for the pH value. While most results are close to 8.9, some results differing by 1 pH unit were received as well.

8.3.7 Statistical evaluation

The datasets accepted on technical grounds and showing sufficient agreement were tested using the Grubbs test for outlying means and using the Cochran test for outlying standard deviations (both at a 99 % confidence level), as well as for normality of dataset means using normal probability plots. Standard deviation within (s_{within}) and between ($s_{between}$) laboratories were calculated using one-way ANOVA. The results of these evaluations are shown in Table 9 for the following measurands:

- CLS: extinction intensity based modal stoked diameter as obtained by CLS according to ISO 13318, line start method
- EM: number weighted modal diameter as obtained by electron microscopy
- DLS: scattering intensity weighted harmonic mean diameter as obtained by ISO 22412, method of cumulants
- zeta-potential as calculated from electrophoretic mobility
- pH as measured by potentiometry using commercial pH electrodes

Table 9: Statistical evaluation of the technically accepted datasets. *p*: number of accepted data sets of results; *s*: standard deviation of the dataset means. Tests for outliers were performed on a 99 % confidence level.

Measurand /method	*p*	Outliers		Normally distributed	Statistical parameters			
		Means	Variance		Average	*s*	$s_{between}$	s_{within}
CLS	6	none	Lab 4 and 5	yes	32.95 nm	1.21 nm	1.10 nm	0.82 nm
EM	11	none	Lab 20	yes	27.82 nm	2.30 nm	2.26 nm	0.96 nm
DLS_{cc}	13	none	none	yes	42.12 nm	0.38 nm	0.28 nm	0.36 nm
ELS	9	none	Lab 13, 21, 22 and 27	yes	-48.48 mV	4.34 mV	4.04 mV	3.89 mV
pH	10	none	Lab 5, 16, 19, 26 and 27	yes	8.80	0.51	0.29	0.50

Outliers of variance show that repeatability varies from laboratory to laboratory. The heterogeneity of variance also prevents pooling all individual results. Further evaluation was based on the mean of laboratory means.

For the intensity-based modal CLS line-start analysis, the distribution has a normal character and no outlying means, however 2 variance outliers occurred. Differences in results ranging from 31 nm to 34 nm are small enough to properly evaluate and assign a certified value.

The number based, modal EM (TEM/SEM) analysis revealed a normal distribution with no outlying mean values but one outlying variance. The data range comprises values from 24 nm to 32 nm. This range of values is very large, which makes an indicative value more appropriate than a certified one.

Laboratory means obtained by DLS follow a normal distribution without outlying means or variances.

The distribution of the ELS data is monomodal and normally distributed with values ranging from -54 mV to -42 mV. No outlying means occurred but four outlying variances were found. The differences in values were considered to be too large as to assign a certified value. The same picture was found for the pH measurements. The values are normally distributed and mono-modal. Values range from 8.0 to 9.8 and the difference is considered to be too large to properly assign a certified value. Instead of certified values, information values have been assigned.

9 Value Assignment

For this material, certified, indicative and information have been assigned.

The certified values for DLS and CLS (see Table 10) are values that fulfil the highest standards of accuracy. Procedures at IRMM require generally pooling of not less than 6 datasets to assign certified values. Full uncertainty budgets in accordance with the Guide to the expression of uncertainty in measurement [23] must be established.

Indicative values like the one for EM (see Table 11) are values where either the uncertainty is deemed too large or where too few independent datasets were available to allow certification. Uncertainties are evaluated according to the same rules as for certified values.

Additional material information like for ζ-potential and pH (see Table 12) refers to values that have been obtained in the course of the study. For example, results reported from only one or two laboratories in cases where individual measurement uncertainty is high, would fall under this category.

9.1 Certified values and their uncertainties

The unweighted means of the means of the accepted datasets for DLS and CLS as shown in Table 9 were used as assigned values for all measurands.

The certified uncertainty consists of uncertainties related to characterisation (u_{char}), between bottle heterogeneity (u_{bb}), degradation during long-term storage (u_{lts}) and during transport to the customer (u_{sts}) [24].

- u_{char} was estimated as the standard error of the mean of laboratory means, i.e. $s/\sqrt{p}$ with s and p taken from Table 9.
- u_{bb} was estimated as the larger value of the standard deviation between-units (s_{bb}) or the maximum heterogeneity potentially hidden by method repeatability (u^*_{bb}). The values are taken from Table 4.
- u_{sts} was estimated from short-term stability tests and was taken from Table 7 (1 week at 60 °C).
- u_{lts} was estimated from long-term stability tests and was taken from Table 7 (24 months of storage at 18 °C).

These uncertainties were regarded as uncorrelated and therefore they were combined quadratically to estimate the standard uncertainty of the certified value (u_{CRM}) as shown in equation 6.

$$u_{CRM} = \sqrt{u_{char}^2 + u_{bb}^2 + u_{lts}^2 + u_{sts}^2} \qquad (6)$$

Certified values were assigned for the measurands shown in Table 10. This table also lists their corresponding uncertainty contributions, as well as the expanded combined uncertainties.

Table 10: Uncertainty budget and certified values

Measurement method/measurand	$u_{char,rel}$ [%]	$u_{bb;rel}$ [%]	$u_{lts,rel}$ [%]	$u_{sts,rel}$ [%]	$u_{CRM,rel}$ [%]	Certified value [nm]	U_{CRM} (k=2) [nm]
DLS, intensity weighted harmonic mean diameter, method of cumulants	0.25	0.41	0.45	0.07	0.66	42.1	0.6
CLS, intensity-based modal Stokes diameter, line-start CLS	1.50	1.35	3.93	0.56	4.45	33.0	3.0

9.2 Indicative value and its uncertainty

Indicative values were assigned for the following measurands and methods:

For the number based modal EM (SEM/TEM) 11 datasets were received. Results were regarded as sufficiently trustworthy to assign a value. However, due to the large spread of results (24 nm to 32 nm), the value was deemed only suitable as indicative value. The same reasoning applies for pH, with results ranging from 8.0 to 9.8.

The principle of uncertainty estimation is the same as for certified values. Uncertainties related to characterisation (u_{char}), between bottle heterogeneity (u_{bb}), degradation during long-term storage (u_{lts}) and during transport to the customer (u_{sts}) were estimated as follows:

- u_{char} was estimated as the standard error of the mean of laboratory means, i.e. $s/\sqrt{p}$ with s and p taken from Table 9.
- u_{bb} was estimated as $u_{rec,rel}$ for DLS from Table 4. The reason for this is that due to the superior repeatability of DLS over CLS this uncertainty is the best estimate for variation in between-unit particle size. This uncertainty therefore also applies to the particle size distribution by EM.
- u_{sts} was estimated from short-term stability tests by DLS and was taken from Table 7 (1 week at 60 °C).
- u_{lts} was estimated from long-term stability tests by DLS and was taken from Table 7 (24 months of storage at 18 °C).

The uncertainty budget was set up as for the certified values and is listed together with the assigned value in Table 11.

Table 11: Uncertainty budget and indicative value

Measurement method	$u_{char,rel}$ [%]	$u_{bb,rel}$ [%]	$u_{lts,rel}$ [%]	$u_{sts,rel}$ [%]	$u_{ind,rel}$ [%]	Indicative value	U_{ind} (k=2)
EM, Number based modal diameter	2.49	0.41	0.45	0.07	2.56	27.8 nm	1.5 nm

9.3 Additional material information

As mentioned in section 8.1, the results for ζ-potential of the initial intercomparison used for selecting qualified laboratories scattered too much to be used as demonstration of competence. Therefore, it was decided from the onset of the study that the results of ζ-potential as determined by ELS could be indicative at most. However, as no values are available for the short- and long-term stability of pH and ζ-potential, the results can be information values only. Therefore, the following information values were assigned:

Table 12: Additional material information

Measurement method	Information value
ELS, ζ-potential	-48 mV
pH	8.8

10 Metrological traceability and commutability

Measurement results with the same established metrological traceability can be compared independently wherever and whenever they are obtained.

The scattering intensity or volume-weighted harmonic mean size of the DLS method is operationally defined by ISO 22412:2008 [17] and ISO 13321:1996 [18]. As DLS is an absolute method and does not need calibration with a calibrant, the measurement results expressed in [nm], are traceable to SI via the monochromatic wavelength of the laser light (Annex C).

The measurand of the line-start CLS method is the extinction intensity based modal Stokes diameter and is operationally defined in the ISO standards 13318-1:2001 [5] and 13318-2:2007 [6] assuming a particle density of be 2.305 g/cm^3. The quantity value is expressed in nanometre [nm]. The results are SI traceable, as calibrants are used with SI traceable certified values. The types of calibrants used in each laboratory are shown in Annex C.

The particle diameter obtained with the EM (SEM/TEM) method is SI traceable through the calibrants used and presented in Annex C.

As the values for ERM-FD304 were obtained from the methods routinely applied, the CRM is by definition commutable.

11 Instructions for use

11.1 Storage conditions

The material shall be stored at 18 ± 5 °C. Ampoules should not be allowed to freeze, as this will irreversibly compromise the integrity of the material.

Please note that the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially of opened samples.

11.2 Safety and protection for the environment

The usual laboratory safety measures apply.

This material should be handled with care. Nanoparticles might have an impact on environment and human health. Any spilling of the suspension should be handled according to the usual laboratory safety precautions.

11.3 Preparation and use of the material

Before opening the ampoule, it should be gently inverted several times to ensure the homogeneity of the suspension and re-suspension of any settled particles. If some suspension is still present in the upper portion of the ampoule (the nipple), it can be removed by gently flicking the nipple with the forefinger while tilting the ampoule. The ampoule is pre-scored and can be opened by applying moderate pressure with one's thumb to snap off the nipple. The contents of an ampoule should be used the same day as opened (except for zeta-potential and pH measurements; see below) without any dilution (except for EM studies; see below).

DLS method: The use of quartz cuvettes is recommended for the measurement. Manual adjustment of the measurement position to the middle of the cell may be needed before applying the DLS method (cumulants).

CLS method: A density of 2.305 g/cm^3 was taken for the evaluation of the results. This number should be used in laboratory calculations and instrument procedure set-up.

EM method (TEM/SEM): A drop of the sample should be put on a holder/grid; after drying at least 500 particles should be measured. If necessary the sample can be diluted with distilled water.

Zeta potential and pH should be measured immediately after opening (storage in air affects the pH and therefore also zeta potential).

11.4 Minimum sample intake

The minimum sample intake representative is 170 μL for CLS and DLS and 3 μL for EM (provided that at least 500 individual particles are measured).

11.5 Use of the certified value

The intended use of this material is to check the performance of instruments and/or methods that characterise the particle size distribution of nanoparticles (particle size ranging from approximately 1 nm to approximately 100 nm) suspended in a liquid medium.

Comparing an analytical result with the certified value.

A result is unbiased if the combined uncertainty of measurement and certified value covers the difference between the certified value and the measurement result (see also ERM Application Note 1 [25]).

Use in quality control charts.

The materials can be used for quality control charts. Different CRM units will give the same result as heterogeneity was included in the uncertainties of the certified values.

Use as a calibrant.

This material can be used as calibrant for methods for which it has certified values. The uncertainty of the certified value shall be taken into account in the final estimation of measurement uncertainty.

12 Acknowledgments

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14 Annexes

Annex A: Results of the homogeneity studies

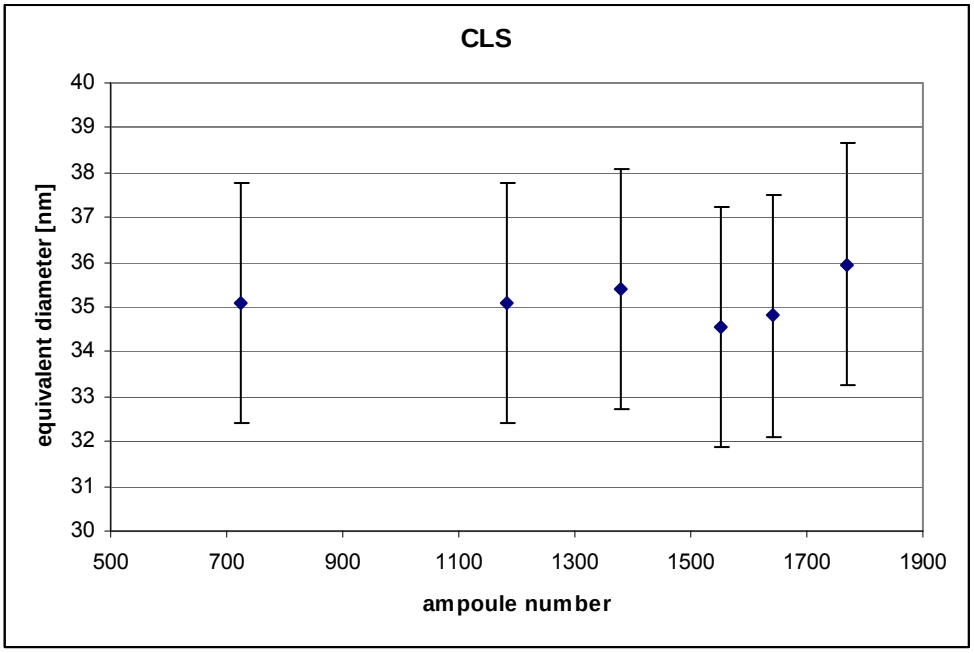
Annex B: Results of the of stability studies

Annex C: Methods and instruments used for characterisation

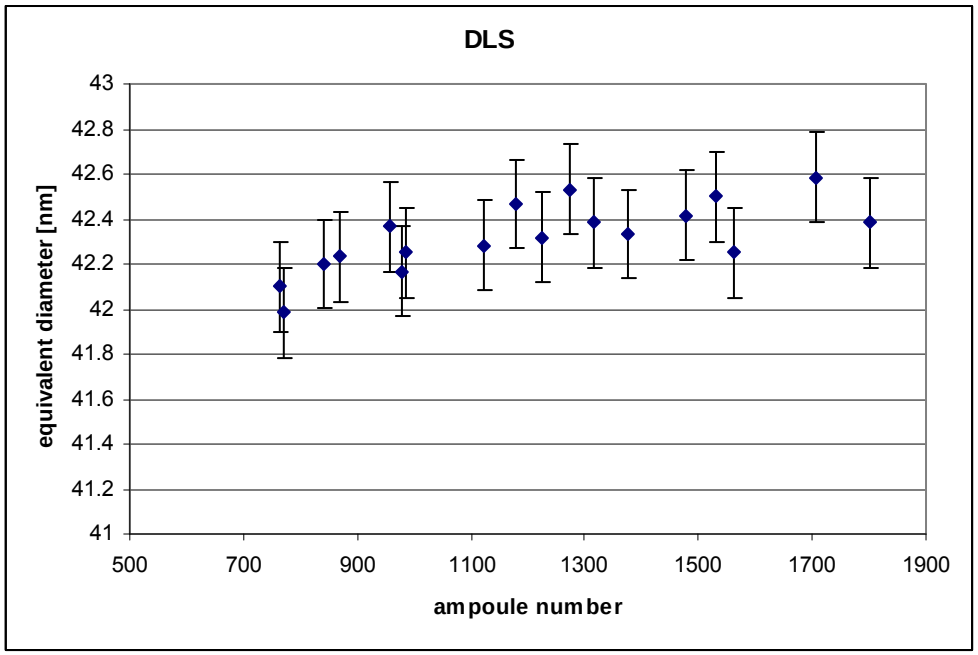
Annex D: Results of the characterisation study

14.1 Annex A: Results of the homogeneity studies

The graphs show ampoule averages and their 95 % confidence intervals of the ampoule means. The same confidence intervals based on the "within-bottle" standard deviation as obtained by ANOVA for each method were assigned to all mean values. Absolute values do not necessarily agree with the certified values due to potential laboratory bias, which is irrelevant for the evaluation of homogeneity.



Annex A figure 1: Results of the homogeneity study by CLS

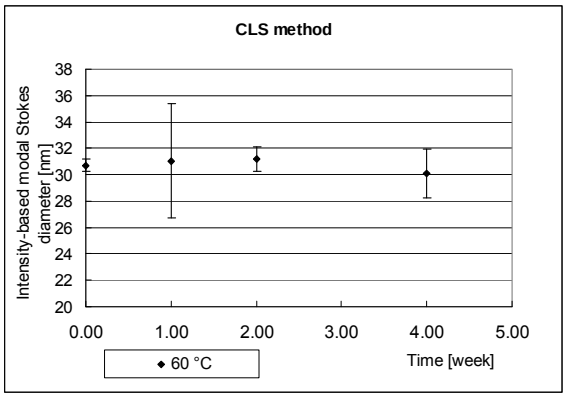


Annex A figure 2: Results of the homogeneity study by DLS

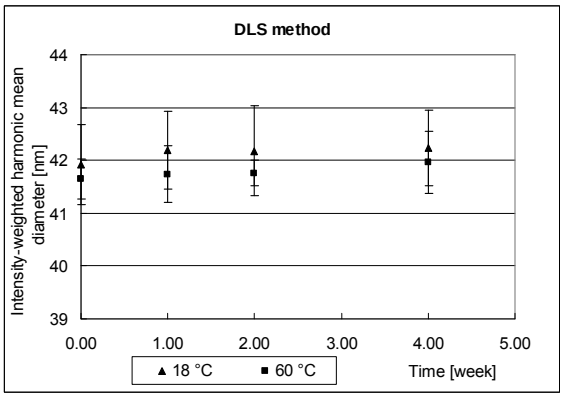
14.2 Annex B: Results of the stability studies

Short-term stability studies

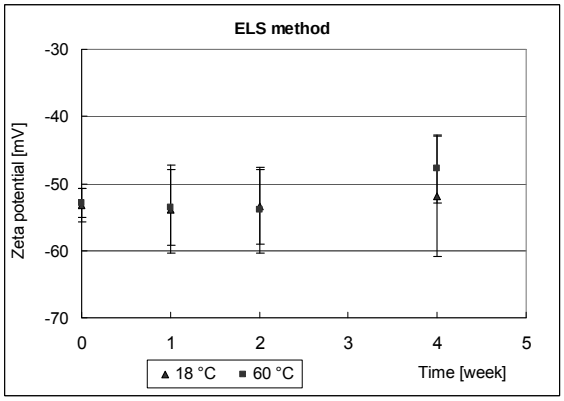
The graphs show averages per time point and their 95 % confidence intervals of the mean of the 4 replicates per time (same value for all points). Confidence intervals are based on the standard deviations of the 4 replicates for DLS and 2 replicates for CLS per time/temperature combination. Data for different temperatures were measured at the same time but are graphically separated to make the graphs easier to read. Only one set of results per method was measured for t = 0, which was entered three times in the graphs. Absolute values do not necessarily agree with the certified values due to potential laboratory bias, which is irrelevant for the evaluation of stability.



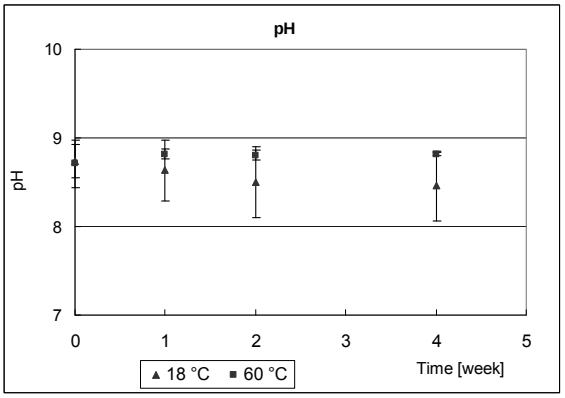
Annex B figure 1:
short-term stability data for CLS



Annex B figure 2
short-term stability data for DLS



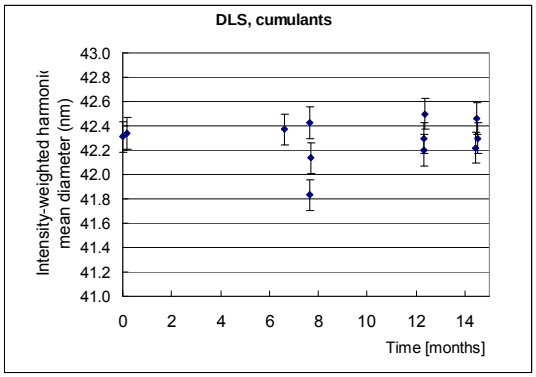
Annex B figure 3:
short-term stability data for ELS



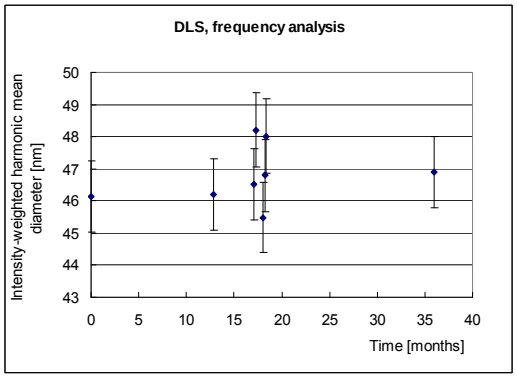
Annex B figure 4
short-term stability data for pH

Long term stability studies

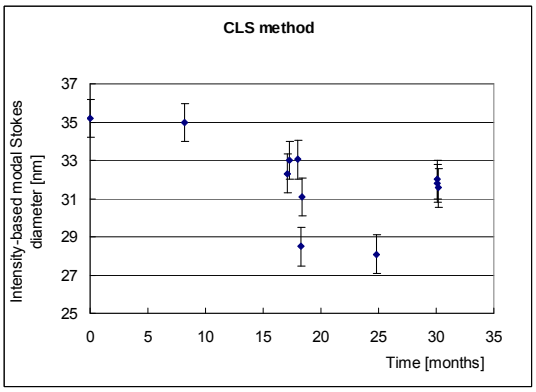
The graphs show averages per time point and their 95 % confidence intervals of the mean of the 16 replicates per time (same value for all points). Confidence intervals are based on the standard deviations of the 16 replicates per time/temperature combination. Absolute values do not necessarily agree with the certified values due to potential laboratory bias, which is irrelevant for the evaluation of homogeneity.



Annex B figure 5:
long-term stability data for DLS, methods of cumulants



Annex B figure 6
long-term stability data for DLS, frequency analysis



Annex B figure 7:
lhort-term stability data for CLS

14.3 Annex C: Instruments and methods used for the characterisation

DLS instruments and methods as reported by the participants

Lab code	Instrument details	Analysis type/data interpretation	Scattering angle [°]	Wavelength [nm]	Reported distribution form	Sample preparation	Sample intake [mL]	Temp. [°C]	Dynamic Viscosity (mPa·s)
5	Particle Sizing System, Nicomp DLS	Correlation function, method of cumulants	90	632.8	intensity	measured as received	0.7	23	0.9
6	Beckman Coulter, Nanosizer N 4+	Correlation function, other methods	90	632.8	Intensity	measured as received	2.5	25	0.9
10a	Malvern, Zetasizer Nano ZS	Correlation function, method of cumulants	173	632.8	intensity and volume	measured as received	4	25	0.9
10b	Horiba, LB-550	Frequency analysis	177	650	intensity and volume	measured as received	4	24	0.8949-0.9566
11	Malvern, Zetasizer Nano ZS	Correlation function, method of cumulants	173	633	intensity and volume	measured as received	2.5	25	0.8872
12	ALV, CGS-3	Correlation function, other methdos	90	632.8	Intensity	measured as received	1.5	21,0-21.59	0.9640-0.9776
13	Microtrac, Nanotrac	Frequency analysis	180	780	intensity and volume	measured as received	2.5	20.8-24.2	0.9-1.0
14	Horiba, LB-550	Frequency analysis	180	650	intensity and volume	measured as received	3.5	20	1
15	Sympatec, Nanophox	Correlation function, method of cumulants	90	632.8	intensity and volume	measured as received	2	20.0-20.1	1
16	Malvern, Zetasizer Nano ZS	Correlation function, method of cumulants	173	633	intensity and volume	measured as received	1	25	0.8872
17a	Sympatec, Nanophox	Correlation function, other methods	90	632.8	intensity and volume	measured as received	2.5	25	0.89
17b	Malvern, HPPS	Correlation function, other methods	173	632.8	intensity and volume	measured as received	2.5	25	0.89

Lab code	Instrument details	Analysis type/data interpretation	Scattering angle [°]	Wavelength [nm]	Reported distribution form	Sample preparation	Sample intake [mL]	Temp. [°C]	Dynamic Viscosity (mPa·s)
18a	Malvern, Zetasizer Nano ZS	Correlation function, other methdos	173	632.8	intensity and volume	measured as received	1.5	25	0.89
18b	Sympatec, Nanophox	Correlation function, other methdos s	90	632.8	intensity and volume	measured as received	1.5	25	0.89
19	Malvern, Zetasizer Nano ZS	Correlation function, method of cumulants	173	632.8	intensity and volume	measured as received	1.5	25	0.8872
20	Precision Detectors, PDEXPERT	Correlation function, other methods	90	658	intensity	measured as received	0.5	24.4	0.9
21	Malvern, Zetasizer Nano	Correlation function, method of cumulants	173	633	intensity and volume	measured as received	1.5	25	0.9
22	Malvern, Zetasizer Nano ZS	Correlation function, method of cumulants	173	633	intensity and volume	measured as received	0.1	25	0.89
23	Horiba instruments, Dynamic Light Scattering	Frequency analysis	180	650	intensity and volume	measured as received	3	21.8-23.1	0.9-1.0
24	Malvern, Zetasizer Nano ZS	Correlation function, other methdos	173	633	intensity and volume	measured as received	2.5	25	0.9
25	Sympatec, Nanophox	Correlation function, method of cumulants	90	632.8	intensity and volume	measured as received	2.5	25	0.9
26	Malvern, Zetasizer Nano ZS	Correlation function, method of cumulants	173	633	intensity and volume	measured as received	1	25	0.9
27	Malvern, Zetasizer nanoseries	Correlation function, method of cumulants	173	633	intensity and volume	measured as received	0.4	25	0.9

CLS instruments and methods

Lab code	Instrument properties						Sample preparation	Calibrant			Sucrose solution		Lab Temp. [°C]
	Instrument details	Analysis type	Rotational speed [rpm]	Laser wavelength [nm]	Sample volume [µL]	Reported distribution form		Type/ Manufacturer	Density [g/cm^3]	Certified value [nm]	Low density [m/m]	High density [m/m]	
1	LUMiSizer 611, LUM GmbH	homog.	4000	470	164 -168	intensity and volume	measured as received	-	-	-	-	-	-
2	XLI ProteomeLab , Beckman	homog.	8000	635	300	volume, no intensity	measured as received	-	-	-	-	-	23 °C
3	DC24000, CPSInstruments	line-start	24000	405	300	intensity and volume	measured as received	PVC calibration standard, CPS Instruments Inc.	1.385	460 ± 18.85	8	24	23 °C
4	DC20000, CPS Instruments	line-start	20000	470	1000	intensity and volume	measured as received	PVC calibration standard, CPS Instruments Inc.	1.385	377	8	24	21 °C
5	DC24000, CPS Instruments	line-start	24000	470	400	intensity and volume	measured as received	PVC calibration standard	1.385	377 ± 12	0	8	23 °C
6	CPS DC24000,	line-start	24000	405	600	intensity and volume	measured as received	Duke scientific	1.83	490 ± 20	8	24	23 °C
7	LUMiSizer 6110-19, L.U.M. GmbH	homog.	4000	470	164 -168	intensity and volume	measured as received	-	-	-	-	-	-
8	Optima XL-1 AUC, Beckman Coulter, Palo Alto	homog.	10000/ 20000	675	370	intensity and volume	measured as received	-	-	-	-	-	25 °C
9	DC24000, CPS Instruments	line-start	24000	470	200	intensity and volume	measured as received	Duke scientific	1.05	300 ± 5.1	2	15	20 °C
10	DC20000, CPS Instruments	line-start	20000	405	200	intensity and volume	measured as received	PVC calibration standard, CPS Instruments Inc.	1.385	460 ± 20	2	8	20 °C

Electron microscopy- SEM/TEM instruments

Lab code	Method/Sample volume intake	Instrument/Software	Sample preparation/drying	Sample grid/sample holder/frame size	Calibration	Particles counted	Ampoules storage temp. (prior to analysis):
3	TEM: micropipette 50 µL	FEI Tecnai 10	no dilution/ 1hour at room T °C in a laboratory hood	Copper grid with carbon layer (300 mesh)	Last PQ on 26/10/2009 with SiO2 particles	500	20 °C
3a	SEM: micropipette 50 µL	Jeol 7500F/ Software SmileView 2.2 and Excel 2003	no dilution/- 1hour at room T °C in a laboratory hood	Gold-coated silicon substrate (25 mm²), frame size 1280*1024 pixels	Last PQ on 15/5/2009	500	20 °C
5	TEM: 5 µL	Philips CM200 STEM/ iTEM by Olympus Soft Imaging Solutions	no dilution/ under vacuum conditions during 2 minutes	Cu-grids, 200 mesh, carbon coated, low background holder; frame size 870x 696 nm	Last PQ 06.05.2010 on grating replica	>500	22 °C
	Comment	In the iTEM-software the particles were measured as a circle, so it was not possible to give a mean aspect ratio.					
11	TEM: 3 µL	Tecnai G2 20 S-TWIN, FEI/- Image J, Origin	volume ratio sample:water=1:10,diluted with MiliQ water and additional filtration with 0.1µm filter size./ 3 h at room temp.	copper grid, carbon film, diameter of the frame 3 mm.	-	500	21 °C
11a	SEM: 3 µL	Hitachi S-4800/ Image J, excel		silicon chip , 4×4 mm	-	500	21 °C
15	TEM: 1 mL	FETEM/JEOL JEM-2100F UHR/ Gatan Digital Micrograph	no dilution/ over 4 hrs and dried in digital dry cabinet	JEOL Double tilt holder (EM-31640), Lacey Formar/Carbon 200mesh Copper grid (Ted Pella No.01881-F),1024 x 1024 Pixels	SPI supplies, Lot 1081229 No.835 (99 ± 1.118 nm)/ Last PQ 2010.03.04	> 500	19±1 °C
15a	SEM: 1 mL	FESEM/JEOL JSM-6500F/ Gatan Digital Micrograph	dilution 1:50 v/v/ over 4 hrs and dried in digital dry cabinet	JEOL SEM holder , 1 drop on copper (QCM) ,1drop on wafer (FD100),1280 x 1027 Pixels	Agar (457.8 ± 2.02 nm)/ Last PQ 010.03.18 on Agar S170A	500	19±1 °C
	Comment	The measurement of ERM-FD100 was performed only by picking particles with the diameter from 10 nm to 30 nm and for QCM only from 20 nm to 50 nm to avoid data from particles overlapping					
16	TEM: 5 µL	TEM Jeol 2100/ ImageJ	dilutions to a target mass fraction of 0.075/ 1 hour in cabinet with nitrogen	EM-11210SQCH Specimen Quick-change holder, 400	NIST 8013 (56 ± 0.5 nm)/ Last PQ on 17/11/2009 on MagICal s/n 988	> 500	20 °C

			atmosphere	Mesh Cu pre coated with lacey/thin carbon film (Pacific Grid Tech)			
20	TEM: 2.5 µL	2000FX JEOL/ ImageJ	dilution 1:50 v/v/ 30 minutes in laboratory bench	JEOL single tilt specimen holder EM-SQH10, carbon coated TEM grids	Agar S106 (463 ± 1 nm)/ Last PQ 03/06/2010 on 2160 lines/mm grating and Catalase crystals.	> 500	18-22 °C
33	TEM: 10 µL	Jeol JEM 1011/ standard software JEOL JEM 1011	no dilution/ excess sample removed, grid air dried at room temp. for 15 min	standard holder of Jeol JEM 1011, Formvar/ carbon 400 mesh copper from EMS	NIST RM 8012 (27.6 ± 2.1 nm)/ Last PQ 19/05/2010 on Grating grid (Pelco 2160 lines/mm) and TMV virus (width 18 nm)	> 500	21 °C
	Comment	Particle sizing was done manual with the software of the JEOL JEM 1011. No automated particle sizing software was used.					
34	TEM: Thies-Weesie dip method	Philips 120CM TEM/ ImageJ	no dilution/ Five minutes on filter paper in a clean room	Carbon coated 200 mesh copper grid	Norrox Scientific Ltd. No. 695 (108.5 ± 2 nm))/ Last PQ 14/4/2010 - 26/4/2010 on MAG*I*CAL	> 500	23 °C

SAXS instruments

Lab code	Instrument details	Electric current [mA]	Type of X-ray	Scattering angular range/resolution	Number of size class	Collimation type	Sample preparation	Sample volume [mL]	Reported distribution form	Mathematical fitting models	Temp. [°C]
28	BESSY II, HZB SAXS at PTB FCM	150-300	0.155 nm (8000 eV)	type 2°/ 0.0015°	n.a.	point	measured as received	0.25	intensity and volume	Gaussian distribution	RT
29	PANalytical B.V., vacuum SAXS camera, Expert SAXS	40	0.1542 nm	0.1-6; 0.05 degrees	100 channels, fit by analytical function	line	measured as received	0.1	intensity and volume	Gaussian distribution	25
30	Anton Paar, SAXSess (Kratky Type)	40	0.1542 nm	0.08 – 6.0 $nm^{-1/}$ 0,002 nm^{-1}	n.a.	line	measured as received	0.04	intensity and volume	polydisperse gauss sphere	25
31	Anton Paar, SAXSess	50	0.1542 nm	0.07-6.3 nm	30-80 nm in steps of 0.7 nm	line	measured as received	0.1	intensity and volume	Inverse RDG transform	25
32	Rigaku, small Angle X-ray Scattering	0.66	0.154 nm	0.007 - 0.16 [A-1]	n.a.	point	measured as received	n.a.	intensity	Guinier	21.7

ELS instruments

Lab code	Instrument details	Laser power [mV]	Wavelength of laser [nm]	Detector type	Detector scattering angle [°]	Sample preparation	Temperature [°C]	Dynamic viscosity [mPa·s]
5	Malvern, Zetasizer Nano ZS	4	633	APD	13	measured as received	25	0.8872
10	Malvern, Zetasizer Nano ZS	4	633	APD	173	measured as received	25	0.8865-0.8879
11	Malvern, Zetasizer Nano ZS	4	633	APD	13	measured as received	n.a.	n.a.
13	Particle Metrix GmbH, ZetaView PMX 100, S/N 117	5	650	Video camera	90	measured as received	21.7-25.5	0.8810-0.9500
16	Malvern, Zetasizer Nano ZS	4	633	APD	173	measured as received	25	0.8904
19	Malvern, Zetasizer Nano ZS, MAL 1029404	4	632.8	APD	12.8	measured as received	25	0.8872
21	Malvern, Zetasizer 3000 HS	5	633	APD	12.8	measured as received	25	0.89
22	Malvern, Zetasizer Nano ZS	4	633	APD	173	measured as received	25	0.8904
26	Malvern, Zetasizer Nano ZS	4	633	APD	173	measured as received	25	0.8872
27	Malvern, Zetasizer Nano ZS	4	633	APD	173	measured as received	25	0.8862-0.8883

pH instruments

Lab code	Instrument details	Instrument type and number	Type of electrode	Calibration range	Standard or reference material used for calibration
5	Horiba	Twin pH-meter B213	Glass-electrode	pH 4 and pH 10	Titrisol 9884,1.09887,9890
10	Metrohm Switzerland	1,7440010/18441	Glass-electrode	pH 4 and pH 9	Buffer Solution pH 7
11	METTLER TOLEDO	FE20	LE438	pH 4 and pH 6.86	Potassium Hydrogen Phthalate (pH 4.00) and Mixed Phosphate (pH 6.86)
13	WTW	PH/cond 340i	SenTix 81 Platin Ceramics Glass membrane	pH 4 and pH 7	DM Messtechnik buffer solutions
16	Cole-Parmer OR Malvern Zetasizer	pH 500 series		pH 7 and pH 10	Oakton buffers, (pH 7) and (pH 10)
19	Oaklon Instruments	pHTestr	Double Junction	pH 4 and pH 10	buffer solutions, pH = 4, 7, 10
21	Thermo Electron Corporation	Orion 3 STAR	InLab@Semi-micro, Mettler Toledo	pH 4 and pH 12	Buffer Solution pH 7
22	Thermo	Orion 410A+	Ag/AgCl	pH 4, pH 7and pH 10	Fisher Sci Buffer Solution pH 4, 7, and 10
26	Accumet	AR25	Glass combination	pH 2 and pH 10.01	Buffers, pH 2, 4.01, 7.01 & 10.01
27	Shindengen	ISFET pH meter KS723	ISFET	pH 1 and pH 14	Commercial buffers provided with electrode

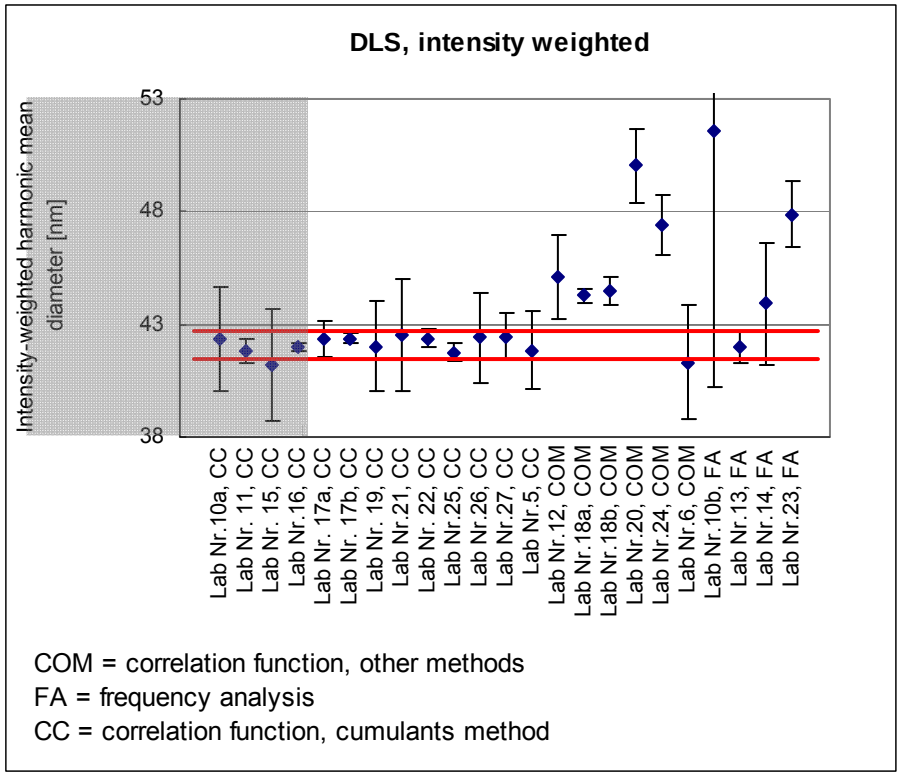
14.4 Annex D: Results of the characterisation study

The graphs show expanded uncertainties as reported from the laboratories, not standard deviations. Results with a low standard deviation may well have a large uncertainty. Laboratories 11 and 12 did not report any uncertainties for the DLS results. The uncertainty was calculated and given as the confidence interval (CI) of a mean for 95 % probability and degrees of freedom (n-1). The red lines in the graphs mark the intervals of the assigned values obtained with each method. This is the assigned value ± expanded uncertainty (k = 2).

Annex D1: Intensity-weighted harmonic mean diameter by DLS

(cc: cumulants; COM: correlation function, other methods, FA: frequency analysis). Grey shaded data were not used for the value assignment.

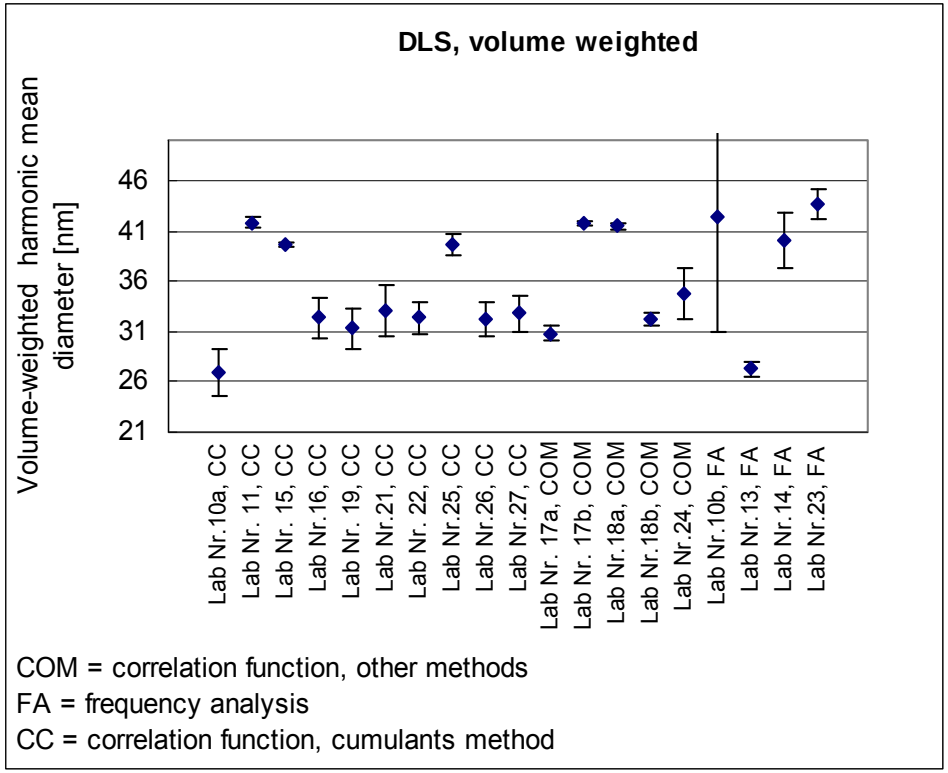
	Average [nm]	s [nm]	U (k=2) [nm]
Lab Nr. 5, CC	41.856	0.138	1.70
Lab Nr. 10a, CC	42.333	0.128	2.30
Lab Nr. 11, CC	41.836	0.275	0.55
Lab Nr. 15, CC	41.211	0.317	2.50
Lab Nr. 16, CC	41.989	0.474	0.20
Lab Nr. 17a, CC	42.378	0.403	0.80
Lab Nr. 17b, CC	42.383	0.214	0.20
Lab Nr. 19, CC	42.000	0.047	2.00
Lab Nr. 21, CC	42.511	0.093	2.51
Lab Nr. 22, CC	42.383	0.105	0.40
Lab Nr. 25, CC	41.772	0.452	0.43
Lab Nr. 26, CC	42.406	0.173	2.00
Lab Nr. 27, CC	42.478	0.323	1.01
Lab Nr. 6, COM	41.328	0.782	2.50
Lab Nr. 12, COM	45.094	0.929	1.86
Lab Nr. 18a, COM	44.261	0.414	0.29
Lab Nr.18b, COM	44.483	0.626	0.61
Lab Nr. 20, COM	50.028	0.387	1.60
Lab Nr. 24, COM	47.433	1.167	1.33
Lab Nr. 10b, FA	51.589	1.79	11.36
Lab Nr. 13, FA	41.978	1.615	0.70
Lab Nr. 14, FA	43.928	0.279	2.70
Lab Nr. 23, FA	47.894	0.334	1.50



Annex D2: Volume-weighted harmonic mean diameter by DLS

(cc: cumulants; COM: correlation function, other methods, FA: frequency analysis). The results depend strongly on the evaluation method, which is subject to further standardisation efforts.

	Average [nm]	s [nm]	U (k=2) [nm]
Lab Nr. 10a, CC	26.90	2.11	2.30
Lab Nr. 11, CC	41.84	0.28	0.56
Lab Nr. 15, CC	39.66	1.24	0.80
Lab Nr. 16, CC	32.39	1.46	0.20
Lab Nr. 19, CC	31.29	0.93	2.00
Lab Nr. 21, CC	33.03	0.85	2.51
Lab Nr. 22, CC	32.34	0.94	1.50
Lab Nr. 25, CC	39.66	0.77	2.00
Lab Nr. 26, CC	32.18	0.69	1.01
Lab Nr. 27, CC	32.77	6.10	2.50
Lab Nr. 17a, COM	30.83	1.04	0.80
Lab Nr. 17b, COM	41.77	0.34	0.20
Lab Nr. 18a, COM	41.46	0.47	0.29
Lab Nr. 18b, COM	32.28	1.35	0.61
Lab Nr. 24, COM	34.73	1.50	1.33
Lab Nr. 10b, FA	42.30	1.62	11.36
Lab Nr. 13, FA	27.30	1.20	0.70
Lab Nr. 14, FA	40.08	0.52	2.70
Lab Nr. 23, FA	43.63	1.20	1.50



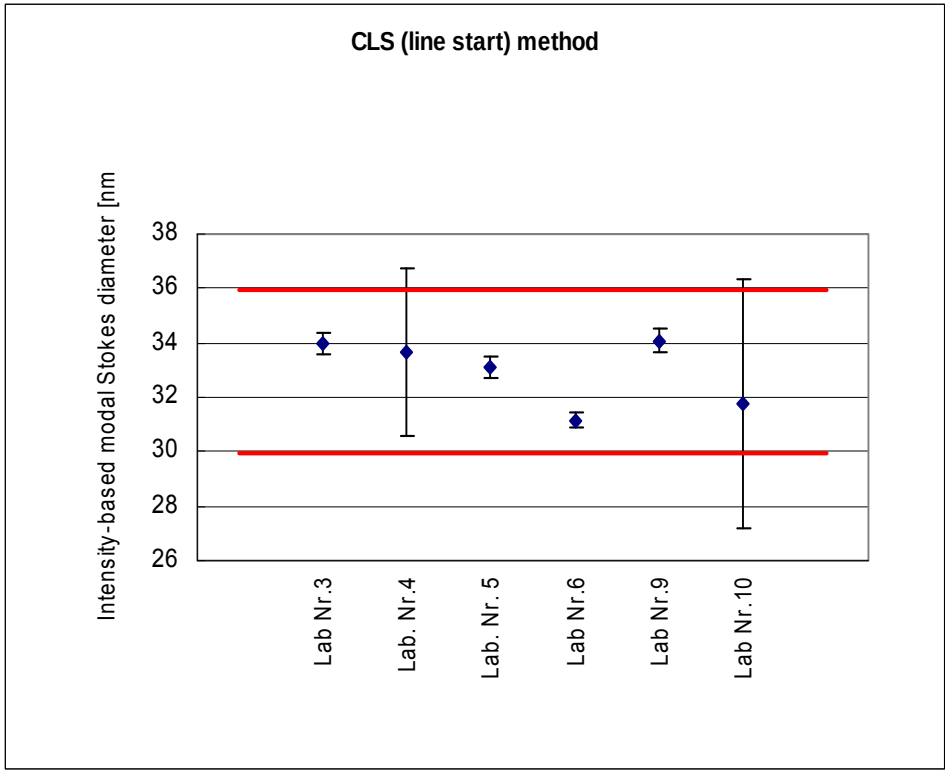
COM = correlation function, other methods

FA = frequency analysis

CC = correlation function, cumulants method

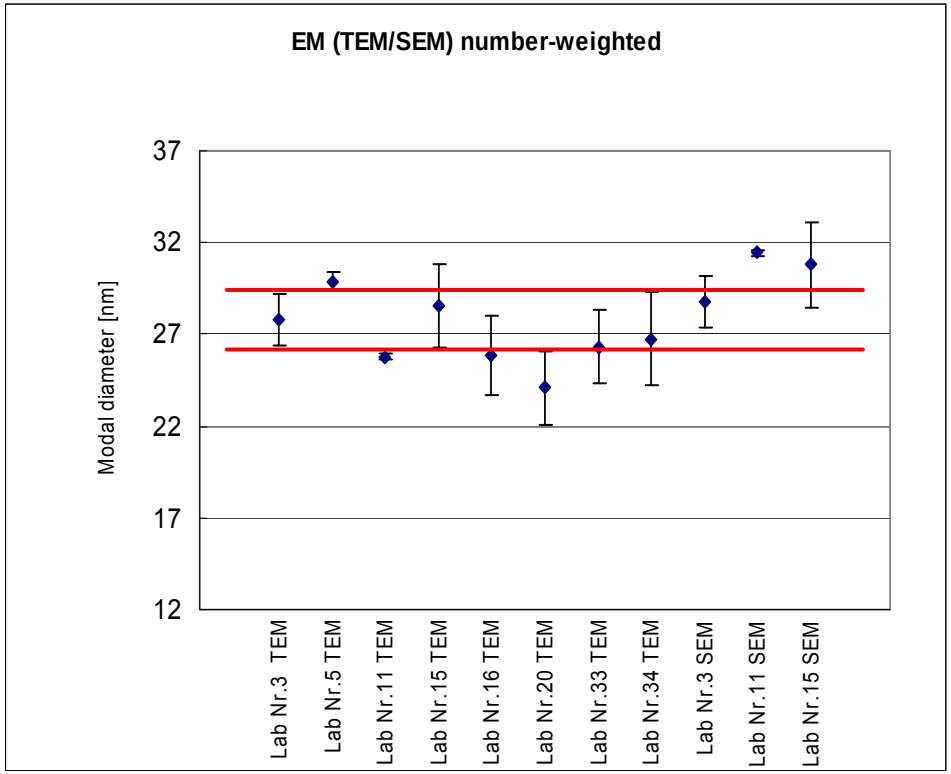
Annex D3: Intensity based modal Stokes diameter (line-start CLS method)

	Average [nm]	s [nm]	U (k=2) [nm]
Lab Nr. 3	33.94	0.18	0.40
Lab. Nr. 4	33.62	1.71	3.08
Lab Nr. 5	33.08	0.71	0.40
Lab Nr. 6	31.16	0.28	0.27
Lab Nr. 9	34.09	0.17	0.40
Lab Nr. 10	31.79	0.29	4.57



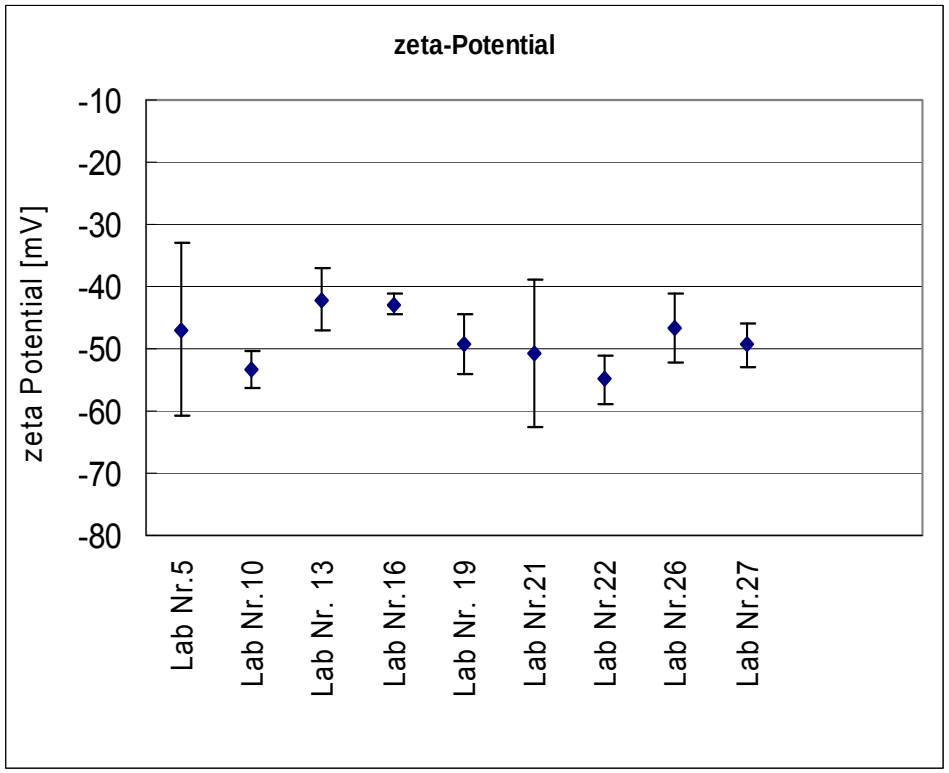
Annex D4: Number based modal diameter TEM/SEM

	Average [nm]	s [nm]	U (k=2) [nm]
Lab Nr. 3 TEM	27.81	1.13	1.40
Lab Nr. 5 TEM	29.89	0.27	0.50
Lab Nr. 11 TEM	25.77	0.21	0.15
Lab Nr. 15 TEM	28.54	0.49	2.30
Lab Nr. 16 TEM	25.89	1.09	2.17
Lab Nr. 20 TEM	24.09	1.05	2.00
Lab Nr. 33 TEM	26.32	0.62	2.00
Lab Nr. 34 TEM	26.76	0.77	2.57
Lab Nr. 3 SEM	28.75	1.29	1.40
Lab Nr. 11 SEM	31.45	1.25	0.15
Lab Nr. 15 SEM	30.78	1.34	2.30



Annex D5: ζ-potential by ELS

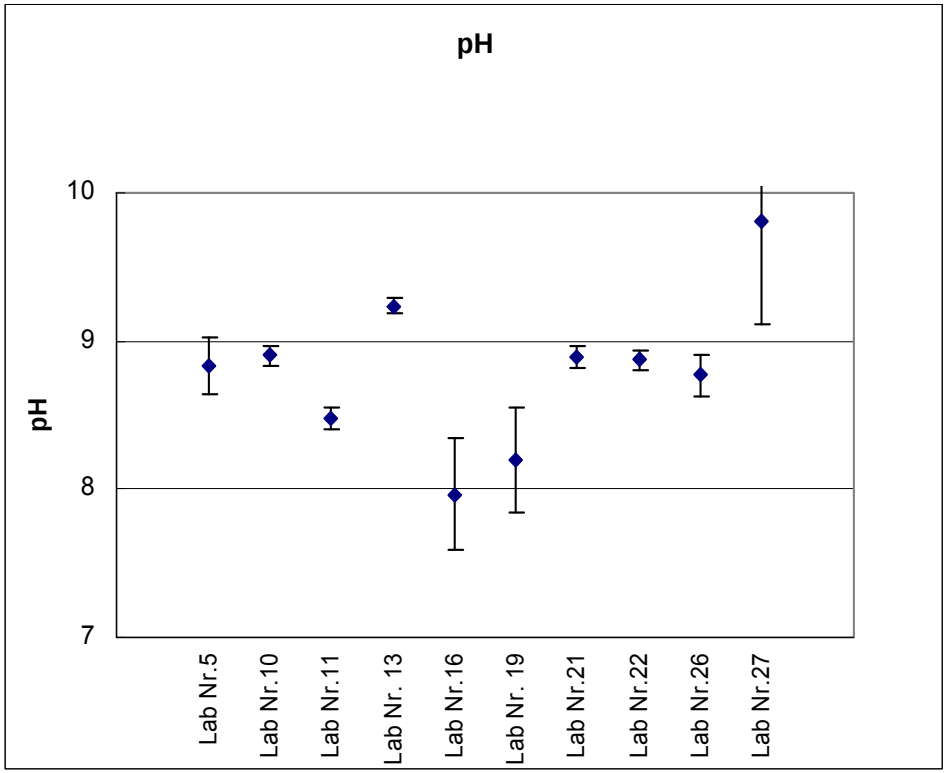
	Average [mV]	s [mV]	U (k=2) [mV]
Lab Nr. 5	-53.44	1.87	13.90
Lab Nr. 10	-42.11	4.00	2.90
Lab Nr. 13	-42.82	0.36	5.00
Lab Nr. 16	-49.12	0.88	1.60
Lab Nr. 19	-50.76	8.66	4.80
Lab Nr. 21	-54.93	5.35	11.70
Lab Nr. 22	-46.70	2.91	4.00
Lab Nr. 26	-49.43	1.37	5.70
Lab Nr. 27	-53.44	1.87	3.50



Annex D6: pH

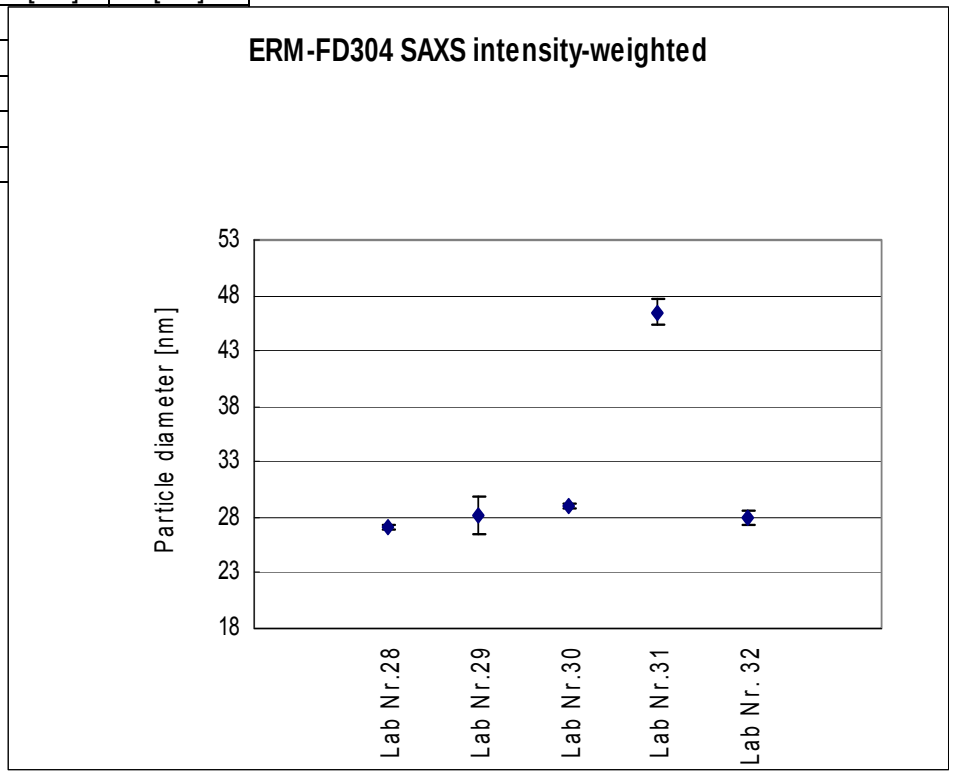
Error bars are standard deviation of the 6 measurements.

	Average	s
Lab Nr.5	8.83	0.19
Lab Nr.10	8.90	0.07
Lab Nr.11	8.48	0.07
Lab Nr. 13	9.23	0.05
Lab Nr.16	7.97	0.37
Lab Nr. 19	8.20	0.36
Lab Nr.21	8.89	0.08
Lab Nr.22	8.87	0.07
Lab Nr.26	8.77	0.14
Lab Nr.27	9.81	0.69



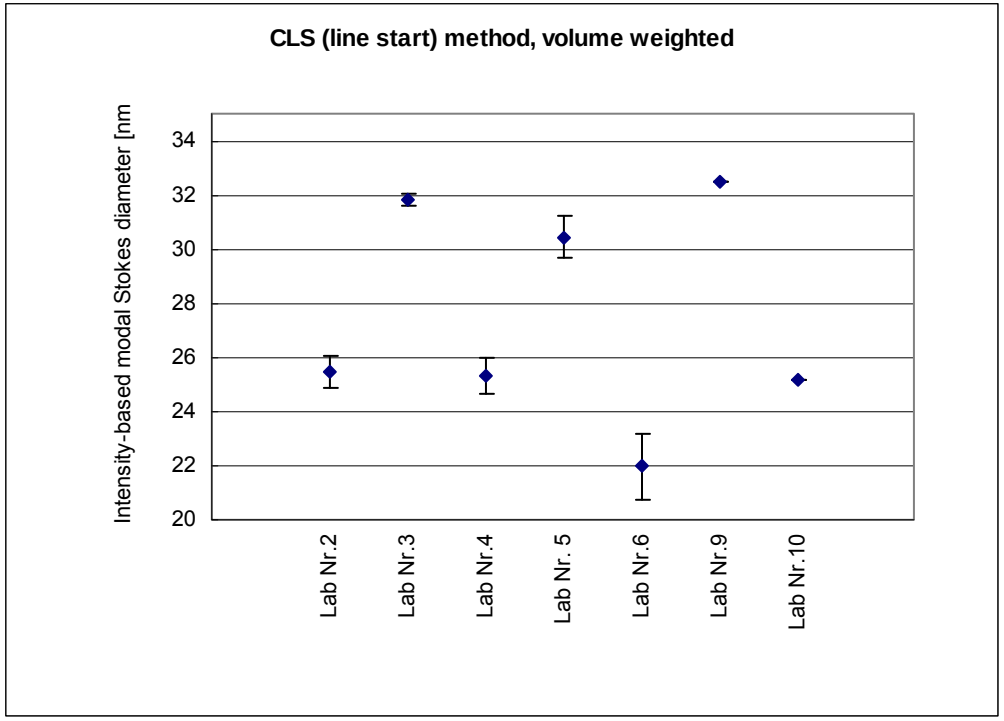
Annex D7: Intensity-weighted mean diameter by SAXS

	Average [nm]	s [nm]	U (k=2) [nm]
Lab Nr. 28	27.16		
Lab Nr. 29	28.10		
Lab Nr. 30	29.00		
Lab Nr. 31	46.52		
Lab Nr. 32	27.96		



Annex D8: Volume based modal Stokes diameter (line-start CLS method)

	Average [nm]	s [nm]	U (k=2) [nm]
Lab Nr.2	25.483	0.497	0.59
Lab Nr.3	31.844	0.293	0.22
Lab Nr.4	25.333	0.728	0.66
Lab Nr. 5	30.444	0.607	0.76
Lab Nr.6	21.961	0.375	1.21
Lab Nr.9	32.5	0.318	not given
Lab Nr.10	25.161	1.15	not given



European Commission

EUR 25018 EN – Joint Research Centre – Institute for Reference Materials and Measurements
Title: Certification of the Equivalent Spherical Diameters of Silica Nanoparticles in Aqueous Solution , Certified Reference Material ERM®-FD304
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Abstract

This report describes the certification of several equivalent spherical diameters of silica nanoparticles in aqueous solution, Certified Reference Material (CRM) ERM®-FD304. The CRM has been certified by the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE.

ERM-FD304 consists of silica nanoparticles suspended in aqueous solution. The intended use of this CRM is to check the performance of instruments and methods that determine the diameter of nanoparticles suspended in liquid medium. The CRM is available in 10 mL pre-scored amber glass ampoules containing approximately 9 mL of suspension.

The CRM was prepared from commercially available colloidal silica, Ludox®, grade TM-50 (Grace Davison GmbH, Worms, Germany)

Production of the CRM included testing of the homogeneity and stability of the ampouled diltuted raw material, as well as the characterisation using an interlaboratory comparison approach.

The CRM has been certified for the scattering intensity based harmonic mean diameter using Dynamic Light Scattering (DLS) and for the extinction intensity based modal Stokes diameter of the silica nanoparticles in aqueous solution using Centrifugal Liquid Sedimentation (CLS) – line start method. An indicative value for size measurements using electron microscopy and information values for pH and ζ-potential were assigned as well. Expanded uncertainties are estimated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) with a coverage factor of k=2, corresponding to a level of confidence of about 95%

The mission of the JRC is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of EU policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Union. Close to the policy-making process, it serves the common interest of the Member States, while being independent of special interests, whether private or national.

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